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(54) Title: MODIFIED FREE-LIVING MICROBES, VACCINE COMPOSITIONS AND METHODS OF USE THEREOF

(57) Abstract: Free-living microbes are provided in which the nucleic acid has been modified so that the attenuated for proliferation and/or which comprise genetic mutations that attenuate the ability of the microbe to repair its nucleic acid. Methods of using the modified microbes for the loading, activation, and/or maturation of antigen-presenting cells are also provided. Vaccine compositions comprising the modified microbes and/or the antigen-presenting cells and methods of using the vaccines are also provided. The microbes may be further modified to include heterologous antigens, such as tumor antigens or infectious disease antigens, for use as a vaccine against cancer or infectious diseases.

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**MODIFIED FREE-LIVING MICROBES, VACCINE COMPOSITIONS AND  
METHODS OF USE THEREOF**

**RELATED APPLICATIONS**

[0001] This application claims the priority benefit of U.S. Provisional Application No. 60/446,051, filed February 6, 2003, U.S. Provisional Application No. 60/449,153, filed February 21, 2003, U.S. Provisional Application No. 60/490,089, filed July 24, 2003, U.S. Provisional Application No. 60/511,869, filed October 15, 2003, the U.S. Provisional Application entitled "Listeria Attenuated for Entry into Non-Phagocytic Cells, Vaccines comprising the Listeria, and Methods of Use Thereof," filed February 2, 2004, the contents of each of which are hereby incorporated by reference into the present disclosure.

**FIELD OF THE INVENTION**

[0001] The present invention relates generally to vaccine compositions and immunotherapy. In particular, the present invention relates to vaccine compositions comprising a population of a modified free-living microbe that can be used to deliver a particular antigen to an individual. In such compositions, the vaccine is directed against the microbe itself or against a heterologous antigen that has been incorporated into the microbe.) The present invention also relates to the use of the modified microbes to load and to induce the activation and maturation of antigen-presenting cells, such as dendritic cells.

**BACKGROUND OF THE INVENTION**

[0002] A variety of vaccines have been developed for clinical use, mostly targeting the prevention of infectious diseases caused by viruses, bacteria and parasites. Vaccines can be prepared from live attenuated microbes, inactivated (killed) microbes, or components of the microbes themselves. Live attenuated microbes contain genetic alterations, such as deletion of virulence factors, resulting in a less virulent microbe. For inactivated vaccines, a microbe may be chemically or physically inactivated. Ideally, such vaccines cannot cause an infection but are still able to stimulate a desired immune

response. Examples of inactivated vaccines include polio and influenza viruses, and bacterial vaccines against cholera and pertussis, although live attenuated vaccines are an option for polio, influenza, and cholera as well. In order to elicit the desired immune response, it is important that the inactivated microbe comprises the appropriate antigens prior to inactivation. It has been observed in some cases that inactivating the microbe results in a significantly reduced immune response because *de novo* gene expression by an infecting microbe is required to stimulate an optimal immune response. This is particularly important for intracellular bacteria. Methods that have been used to inactivate bacteria include the use of acetone, alcohol, formalin, glutaraldehyde, paraformaldehyde, or phenol, heating, or ultraviolet irradiation [Pace et al., Vaccine 16(16):1563 (1998)].

[0003] In addition to using microbial vaccines to prevent infectious diseases caused by the microbe itself, the microbes can be modified to contain heterologous nucleic acid sequences that encode a certain protein or antigen. Such recombinant microbes are used as delivery vehicles and may be used as vaccines to stimulate an immune response to the heterologous antigens. These recombinant vaccines have been shown to be effective in animal models. An oral vaccine of live attenuated *Salmonella* modified to express *Plasmodium berghei* circumsporozoite antigen has been shown to protect mice against malaria [Aggarwal et al., J Exp Med 172(4):1083 (1990)]. Similarly, U.S. Patent No. 6,051,237 describes a live recombinant form of *Listeria monocytogenes* that grows and spreads and expresses a tumor-specific antigen for use as a cancer vaccine. While such recombinant vaccines may be effective, each microbe strain must be genetically modified to provide the vaccine. It would therefore be desirable to develop a method of producing a safe and effective microbial vaccine that can be applied to any microbe, whether or not the microbe comprises recombinant antigens. Dendritic cell (DC)-based immunotherapy has been widely investigated and demonstrated to provide a clinical benefit for the treatment of a wide range of tumor types. A variety of strategies are presently being developed to isolate and generate autologous dendritic cells (DC), and subsequently load them with antigen or peptides *ex vivo* prior to patient vaccination. Recent advances in the understanding of immune mechanisms have, in addition to efficient antigen loading, highlighted the importance of the activation and maturation state of DC used for vaccination on the efficacy of cancer immunotherapy. Whereas immature DC are more effective in the uptake and processing of antigen, activated/mature DC lose this capacity, yet are more potent at presenting antigen to naïve T lymphocytes in the context of MHC molecules. In fact, mature DCs have been found to be potent antigen presenting cells

(APC) to induce primary T lymphocyte responses, overcoming peripheral T cell tolerance and enhance anti-tumor immunity. Despite the development of a variety of methods to load and to stimulate the activation and maturation of DC that has led to encouraging clinical data, there still are not standard efficient and cost effective methods for combining antigen loading with DC activation and maturation.

### SUMMARY OF THE INVENTION

[0004] The invention involves a free-living microbe, in which the proliferation of the microbe is attenuated while maintaining sufficient microbial gene expression, wherein the attenuation can be controlled in a dose-dependent manner. The invention includes methods for this attenuation of the free-living microbe. The invention includes vaccine compositions comprising these attenuated microbes. The present invention also provides novel uses of modified microbes, and attenuated *Listeria*, in particular, to load and to induce the activation and maturation of antigen-presenting cells, such as dendritic cells, *in vitro* or *ex vivo*. The resulting antigen-presenting cells are useful in vaccines and immunotherapy. In particular embodiments, the provided vaccines and immunotherapy are directed against cancer.

[0005] In one aspect, the invention provides a vaccine comprising a free-living microbe, wherein the nucleic acid of the microbe (e.g., genomic nucleic acid) is modified so that the microbe is attenuated for proliferation. In some embodiments, the attenuation of the proliferation of the microbe is controllable in a dose-dependent manner. In some embodiments, microbial gene expression in the microbe is substantially unaffected by attenuation of the proliferation of the microbe. In some embodiments, the microbe in the vaccine expresses an antigen at a sufficient level to induce an immune response to the antigen in an individual upon administration of the vaccine to the individual. In some embodiments, the nucleic acid has been modified by reaction with a nucleic acid targeted compound (alternatively termed a nucleic acid "targeting" compound) which reacts directly with the nucleic acid. In one embodiment, the nucleic acid target compound is an alkylator such as  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, also referred to herein as "S-59") activated by UVA irradiation. In some embodiments, the microbe in the vaccine comprises a genetic



mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified. In some embodiments, the microbe is a bacterium, such as *Bacillus anthracis* or *Listeria monocytogenes*. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. In some embodiments, the vaccine further comprises a pharmaceutically acceptable carrier and/or an adjuvant. The invention further provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the vaccine. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the vaccine, wherein the microbe expresses the antigen.

[0006] In another aspect, the invention provides a vaccine comprising a free-living microbe (e.g., a bacterium) which is defective with respect to at least one DNA repair enzyme. In some embodiments, the free-living microbe comprises a genetic mutation in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*. In some embodiments, the microbe comprises genetic mutations in both *uvrA* and *uvrB* (or in functional equivalents of both *uvrA* and *uvrB*, depending upon the genus and species of the microbe). In some embodiments, the microbe is defective with respect to *RecA* (or the functional equivalent of *RecA*, depending upon the genus and species of the microbe). In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen (for instance, a cancer antigen, or an infectious disease antigen foreign to the microbe). In some embodiments, the vaccine further comprises a pharmaceutically acceptable carrier or an adjuvant. The invention further provides method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the vaccine. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the vaccine wherein the microbe expresses the antigen.

[0007] In another aspect, the invention provides an isolated mutant *Listeria* strain, such as a mutant *Listeria monocytogenes* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the mutant *Listeria* strain is defective with respect to at least one DNA repair enzyme (such as *UvrA* and/or *UvrB*). In some embodiments, the mutant *Listeria* strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the mutant strain is the *Listeria monocytogenes actA/uvrAB* strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563. In other embodiments, the strain

is a mutant of the *Listeria monocytogenes actA/uvrAB* strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563, wherein the mutant of the deposited strain is defective with respect to UvrA, UvrB, and ActA. The invention further provides vaccines and professional antigen-presenting cells comprising the mutant *Listeria* strain. Methods of using the modified *Listeria* strain to induce immune responses and to prevent or treat disease are also provided.

[0008] In another aspect, the invention provides an isolated mutant *Bacillus anthracis* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the mutant strain is defective with respect to at least one DNA repair enzyme (such as UvrA and/or UvrB). In some embodiments, the mutant strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the mutant strain is attenuated with respect to RecA. In some embodiments, the mutant strain comprises a genetic mutation in the *recA* gene. In some embodiments, the mutant strain comprises one or more mutations in the *lef* gene, *cya* gene, or both genes, that decreases the toxicity of the strain. The invention further provides vaccines and professional antigen-presenting cells comprising the mutant strain. Methods of using the modified *Bacillus anthracis* strain to induce immune responses and to prevent or treat disease are also provided.

[0009] In one embodiment, the invention includes a vaccine comprising bacteria that has been reacted with a psoralen compound and UVA light, wherein the proliferation of the bacteria is attenuated. In one embodiment, the bacterial expression is sufficiently active after the psoralen modification such that the psoralen attenuated bacteria can continue to express a protein antigen, wherein when the bacteria is administered to an individual, an immune response to the antigen is elicited. In one embodiment, the desired immune response is to the bacteria itself. In one embodiment, the bacteria are a recombinant strain that expresses a heterologous protein antigen, wherein when the bacteria is administered to an individual, an immune response to the heterologous antigen is elicited. Such a vaccine comprising a heterologous antigen may be designed to treat or prevent a variety of diseases including infectious diseases, autoimmune diseases, allergies, cancers, and other hyperproliferative diseases.

[0010] For the treatment or prevention of infectious diseases, the disease causing agent may be prepared according to the methods of the invention to be used as the vaccine. In one embodiment, a vaccine can be prepared from a microbe of the invention comprising a heterologous antigen from the disease-causing agent, such as a virus, bacterium or

parasite. Such a vaccine may provide a level of benefit when the health risk of receiving the bacterial vector is significantly less than the risks associated with possible infection by the infectious agent. A heterologous vaccine for the treatment or prevention of infectious disease that is attenuated by the methods of the present invention may have other benefits as well. First, it may not be possible to prepare an attenuated live vaccine or a killed vaccine directly from the infectious agent itself. Second, if a live vaccine is required, it may not be possible to otherwise attenuate the infectious agent and still maintain an appropriate immune response.

[0011] Another possibility is that the antigen inserted into the bacterial vector does not stimulate an immune response in an individual in the absence of the innate immune response induced by the bacterial vector. For example, diseases in which autologous cells proliferate improperly may contain antigens that do not typically stimulate an immune response. It may be useful to fight such diseases by finding a way to stimulate such an immune response against an autologous antigen. In one embodiment the proliferating cells express or over express an antigen at higher levels than on a normal cell so that the immune response is largely specific to the proliferating cells. Diseases that may be treated with such a vaccine include, but are not limited to, autoimmune diseases, allergies, cancers and other hyperproliferative cellular diseases. In another embodiment, the vaccine may target a product of the disease or a disease related target rather than the diseased cells themselves. For example, tumors may be treated with a vaccine targeting vascular endothelial growth factor (VEGF), which is essential for generation of new blood vessels required to feed tumor cells. The VEGF is peripheral to the tumor cells themselves but is prevalent in areas of tumor growth and is a viable vaccine target that could potentially limit the growth of the tumor cells. Another example is a vaccine that comprises an antigen that will elicit a response to a disease related protein, such as the proteins that cause the amyloid plaques characteristic of Alzheimer's disease or Creutzfeldt-Jakob disease. Similarly, the vaccine may target proteins involved in autoimmune or allergic responses. The vaccine may comprise an idio~~type~~ antigen that can elicit a response to the specific antibodies or cells, such as B-cells or T cells, causing an autoimmune or allergic response.

[0012] In one embodiment, the invention includes a vaccine composition comprising a free-living microbial population in which the microbial nucleic acid is modified so that the proliferation of the microbial population is attenuated, wherein the microbial gene expression is substantially unaffected. In one embodiment, the microbial gene expression is substantially unaffected so that an antigen is expressed at a level

sufficient to stimulate an immune response upon administration of the microbial population to an individual. In one embodiment, the proliferation of the microbial population is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the microbial population is attenuated by about 0.3 to > 10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In one embodiment, the expression of an antigen by the microbial population is at least about 10%, about 25%, about 50%, about 75%, or at least about 90% of the expression of the antigen by a microbial population in which the microbial nucleic acid is not modified. In one embodiment, the antigen expressed is an antigen from the microbe itself. In one embodiment, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. In one embodiment, the antigen is a disease associated antigen. In one embodiment, the antigen is associated with a disease selected from the group consisting of infectious diseases, autoimmune diseases, allergies, cancers, and other hyperproliferative diseases. In one embodiment, the antigen is a tumor associated antigen. In one embodiment, the tumor antigen is selected from the group consisting of differentiation antigens, tissue-specific antigens, developmental antigens, tumor-associated viral antigens, cancer-testis antigens, embryonic antigens, oncoprotein antigens, over-expressed protein antigens and mutated protein antigens. In one embodiment, the tumor antigen is selected from the group consisting of mesothelin, Sp17, gp100, EphA2, PR3, PAGE-4, TARP, and SPAS-1. In one embodiment, the microbial nucleic acid is modified by a method selected from the group consisting of exposing the microbe to radiation and reacting the microbe with a nucleic acid targeted compound that causes the modification of the microbial nucleic acid. In a preferred embodiment, the microbial nucleic acid is modified by reacting the microbial population with a nucleic acid targeted compound that reacts directly with the nucleic acid. In one embodiment, the nucleic acid targeted compound is targeted to the nucleic acid by a mode selected from the group consisting of intercalation, minor groove binding, major groove binding, electrostatic binding, and sequence-specific binding. In one embodiment, the nucleic acid targeted compound comprises a nucleic acid alkylator. In a preferred embodiment, the nucleic acid targeted compound is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In one embodiment, the nucleic acid targeted compound that reacts directly with the nucleic acid reacts upon activation of the compound by irradiation, preferably by UVA irradiation. In

one embodiment, the nucleic acid targeted compound activated by UVA irradiation is a psoralen. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the nucleic acid targeted compound indirectly causes the modification of the nucleic acid. In one embodiment, the nucleic acid targeted compound indirectly causes modification upon activation by irradiation, preferably by UVA irradiation. In one embodiment, the microbe comprises a genetic mutation. In one embodiment, the genetic mutation results in the attenuation of the ability of the microbe to repair microbial nucleic acid that has been modified. In one embodiment, the genetic mutation is in the gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the microbe. In one embodiment, the genetic mutation is in more than one of the genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the microbe. In an embodiment where the mutation is in the *recA* gene, whether alone or in combination with one or more other mutations, the *recA* mutation is a conditional mutation. In one embodiment, the genetic mutation results in the attenuation in the activity of at least one of a DNA repair enzyme selected from the group consisting of PhrB, UvrA, UvrB, UvrC, UvrD and RecA. In one embodiment, attenuation in the activity of RecA is conditional. In a further embodiment, the microbes containing these mutations are modified by reaction with a psoralen activated by UVA irradiation. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the microbe is selected from the group consisting of bacteria, protozoa and fungi. In one embodiment, the microbe is a bacteria. In one embodiment, the bacteria is a mycobacteria. In one embodiment, the mycobacteria is *Mycobacterium tuberculosis*. In one embodiment, the bacteria is an intracellular bacteria. In one embodiment, the intracellular bacteria is *Bacillus anthracis*. In one embodiment, the intracellular bacteria is *Yersinia pestis*. In a preferred embodiment, the bacteria is a *Listeria*, preferably *Listeria monocytogenes*. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to invade non-phagocytic cells without significantly affecting the uptake of the *Listeria* by phagocytic cells. In one embodiment, the *Listeria* mutation is in an internalin gene(s). In one embodiment, the *Listeria* mutation is in the gene selected from the group consisting of *inlA*, *inlB*, and any gene encoding an internalin. In one embodiment, the *Listeria monocytogenes* comprises a genetic mutation in both the *inlA* and *inlB* genes. In one embodiment, the *Listeria* comprises a mutation that results in the

attenuation of the ability of the *Listeria* to escape the phagolysosome of an infected cell. In one embodiment, the *Listeria* mutation is in the *hly* gene. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the polymerization of actin by the *Listeria*. In one embodiment, the *Listeria* mutation is in the *actA* gene. In one embodiment, the *Listeria monocytogenes* comprises more than one mutation. In a preferred embodiment, the *Listeria* mutations are in both the *actA* and *inlB* genes, preferably deletion mutations in both the *actA* and *inlB* genes.

[0013] In one embodiment, the invention includes a vaccine comprising a microbial population in which the microbial nucleic acid is modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the proliferation of the microbial population is attenuated, wherein the microbial gene expression is substantially unaffected, and wherein the microbe of the population comprises a heterologous nucleic acid sequence encoding a tumor antigen. In one embodiment, the microbial gene expression is substantially unaffected so that the tumor antigen is expressed at a level sufficient to stimulate an immune response upon administration of the microbe to an individual. In one embodiment, the proliferation of the microbial population is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the microbial population is attenuated by about 0.3 to > 10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In one embodiment, the expression of the tumor antigen by the microbial population is at least about 10%, about 25%, about 50%, about 75%, or at least about 90 % of the expression of the tumor antigen by a microbial population in which the microbial nucleic acid is not modified. In one embodiment, the tumor antigen is selected from the group consisting of differentiation antigens, tissue-specific antigens, developmental antigens, tumor-associated viral antigens, cancer-testis antigens, embryonic antigens, oncoprotein antigens, over-expressed protein antigens and mutated protein antigens. In one embodiment, the tumor antigen is selected from the group consisting of mesothelin, Sp17, gp100, EphA2, PR3, PAGE-4, TARP, and SPAS-1. In one embodiment, the nucleic acid targeted compound comprises an alkylator. In one embodiment, the alkylator is selected from the group consisting of mustards, mustard intermediates and mustard equivalents. In one embodiment, the nucleic acid targeted compound comprises a nucleic acid targeting group selected from the group consisting of intercalators, minor groove binders, major groove binders, electrostatic binders, and sequence-specific binders. In one embodiment,

the nucleic acid targeted compound is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In one embodiment, the nucleic acid targeted compound reacts directly with the nucleic acid upon activation of the compound. In one embodiment, the activation of the compound is by irradiation. In one embodiment, the irradiation is UVA irradiation. In a preferred embodiment, the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the microbe of the population comprises a genetic mutation. In one embodiment, the genetic mutation results in the attenuation of the ability of the microbe to repair microbial nucleic acid that has been modified. In one embodiment, the genetic mutation is in the gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the microbe. In one embodiment, the genetic mutation is in more than one of the genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the microbe. In an embodiment where the mutation is in the *recA* gene, whether alone or in combination with one or more other mutations, the *recA* mutation is a conditional mutation. In one embodiment, the genetic mutation results in the attenuation in the activity of at least one of a DNA repair enzyme selected from the group consisting of PhrB, UvrA, UvrB, UvrC, UvrD and RecA. In one embodiment, attenuation in the activity of RecA is conditional. In a further embodiment, the microbes containing these mutations are modified by reaction with a psoralen activated by UVA irradiation. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the microbe is selected from the group consisting of bacteria, protozoa and fungi. In one embodiment, the microbe is a bacteria. In one embodiment, the bacteria is an intracellular bacteria. In a preferred embodiment, the bacteria is a *Listeria*, preferably *Listeria monocytogenes*. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to invade non-phagocytic cells without significantly affecting the uptake of the *Listeria* by phagocytic cells. In one embodiment, the *Listeria* mutation is in an internalin gene(s). In one embodiment, the *Listeria* mutation is in the gene selected from the group consisting of *inlA*, *inlB*, and any gene encoding an internalin. In one embodiment, the *Listeria monocytogenes* comprises a genetic mutation in both the *inlA* and *inlB* genes. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to escape the phagolysosome of

an infected cell. In one embodiment, the *Listeria* mutation is in the *hly* gene. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the polymerization of actin by the *Listeria*. In one embodiment, the *Listeria* mutation is in the *actA* gene. In one embodiment, the *Listeria monocytogenes* comprises more than one mutation. In a preferred embodiment, the *Listeria* mutations are in both the *actA* and *inlB* genes, preferably deletion mutations in both the *actA* and *inlB* genes. In a preferred embodiment, the *Listeria monocytogenes actA/inlB* deletion mutant further comprises a deletion mutation in the *uvrAB* gene.

[0014] In one embodiment, the invention includes a vaccine comprising a *Listeria monocytogenes* population in which the listerial nucleic acid is modified by reaction with a psoralen activated by UVA irradiation so that the proliferation of the listerial population is attenuated, wherein the listerial gene expression is substantially unaffected, and wherein the *Listeria monocytogenes* comprises a heterologous nucleic acid sequence encoding a tumor antigen. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the listerial gene expression is substantially unaffected so that the tumor antigen is expressed at a level sufficient to stimulate an immune response upon administration of the *Listeria* to an individual. In one embodiment, the proliferation of the listerial population is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the listerial population is attenuated by about 0.3 to >10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In one embodiment, the expression of the tumor antigen by the listerial population is at least about 10%, about 25%, about 50%, about 75%, or at least about 90% of the expression of the tumor antigen by a listerial population in which the listerial nucleic acid is not modified. In one embodiment, the tumor antigen is selected from the group consisting of differentiation antigens, tissue-specific antigens, developmental antigens, tumor-associated viral antigens, cancer-testis antigens, embryonic antigens, oncoprotein antigens, over-expressed protein antigens and mutated protein antigens. In one embodiment, the tumor antigen is selected from the group consisting of mesothelin, Sp17, gp100, EphA2, PR3, PAGE-4, TARP, and SPAS-1. In one embodiment, the *Listeria monocytogenes* comprises a genetic mutation. In one embodiment, the genetic mutation results in the attenuation of the ability of the *Listeria monocytogenes* to repair nucleic acid that has been modified. In one embodiment, the genetic mutation is in the gene selected from the group consisting of *phrB*, *zvrA*, *uvrB*,



*uvrC*, *uvrD* and *recA*. In one embodiment, the genetic mutation is in more than one of the genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*. In an embodiment where the mutation is in the *recA* gene, whether alone or in combination with one or more other mutations, the *recA* mutation is a conditional mutation. In one embodiment, the genetic mutation results in the attenuation in the activity of at least one of a DNA repair enzyme selected from the group consisting of *PhrB*, *UvrA*, *UvrB*, *UvrC*, *UvrD* and *RecA*. In one embodiment, attenuation in the activity of *RecA* is conditional. In one embodiment, the genetic mutation results in the attenuation of the ability of the *Listeria monocytogenes* to invade non-phagocytic cells without significantly affecting the uptake of the *Listeria monocytogenes* by phagocytic cells. In one embodiment, the genetic mutation is in an internalin gene(s). In one embodiment, the *Listeria* mutation is in the gene selected from the group consisting of *inlA*, *inlB*, and any gene encoding an internalin. In one embodiment, the *Listeria monocytogenes* comprises a genetic mutation in both the *inlA* and *inlB* genes. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to escape the phagolysosome of an infected cell. In one embodiment, the *Listeria* mutation is in the *hly* gene. In one embodiment, the genetic mutation results in the attenuation of the polymerization of actin by the *Listeria*. In one embodiment, the *Listeria* mutation is in the *actA* gene. In one embodiment, the *Listeria monocytogenes* comprises more than one mutation. In a preferred embodiment, the *Listeria* mutations are in both the *actA* and *inlB* genes, preferably deletion mutations in both the *actA* and *inlB* genes. In a preferred embodiment, the *Listeria monocytogenes actA/inlB* deletion mutant further comprises a deletion mutation in the *uvrAB* gene.

[0015] In another aspect, the invention provides a professional antigen-presenting cell (e.g., a dendritic cell) comprising a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. In some embodiments, the attenuation of the proliferation of the microbe is controllable in a dose-dependent manner. In some embodiments, microbial gene expression in the microbe is substantially unaffected by attenuation of the proliferation of the microbe. In some embodiments, the microbe in the vaccine expresses an antigen at a sufficient level to induce an immune response to the antigen in an individual upon administration of the vaccine to the individual. In some embodiments, the nucleic acid has been modified by reaction with a nucleic acid targeted compound which reacts directly with the nucleic acid. In one embodiment, the nucleic acid target compound is an alkylator such as  $\beta$ -alanine, N-(acridin-

9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, also referred to herein as "S-59") activated by UVA irradiation. In some embodiments, the microbe in the vaccine comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified. In some embodiments, the microbe is a bacterium. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. The invention also provides a vaccine comprising the antigen-presenting cell. The invention further provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the antigen-presenting cell. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the antigen-presenting cell, wherein the microbe expresses the antigen. The invention further provides a method of activating naïve T cells *ex vivo* and/or *in vitro* (not mutually exclusive), comprising contacting the naïve T cells with the professional antigen-presenting cell under suitable conditions and for a sufficient time to activate the naïve T-cells.

[0016] In another aspect, the invention provides an isolated professional antigen-presenting cell (e.g., a dendritic cell) comprising a free-living microbe (e.g., a bacterium) which is defective with respect to at least one DNA repair enzyme. In some embodiments, the microbe comprises a genetic mutation in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*. For instance, the microbe may comprise genetic mutations in both *uvrA* and *uvrB*, or in functional equivalents of both *uvrA* and *uvrB* (depending on the genus and species of the microbe). In some embodiments, the antigen-presenting cell is defective with respect to *RecA*, or the functional equivalent of *RecA*. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. Methods of preventing or treating a disease in a host, comprising administering to the host an effective amount of the antigen-presenting cell are also provided, as are methods of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the antigen-presenting cell, wherein the microbe expresses the antigen.

[0017] In another aspect, the invention provides a method of loading professional antigen-presenting cells with an antigen comprising contacting the professional antigen-presenting cells (*in vitro* or *in vivo*) with a free-living microbe that comprises a nucleic acid

sequence encoding the antigen, under suitable conditions and for a sufficient time to load the professional antigen-presenting cells, wherein the nucleic acid of the microbe is modified (e.g., has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid) so that the microbe is attenuated for proliferation.

[0018] In still another aspect, the invention provides a method of activating and/or maturing professional antigen-presenting cells comprising contacting the professional antigen-presenting cells (*in vitro* or *in vivo*) with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, under suitable conditions and for a sufficient time to load the professional antigen-presenting cells, wherein the nucleic acid of the microbe is modified (e.g., has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid) so that the microbe is attenuated for proliferation.

[0019] In yet another aspect, the invention provides a method of preventing or treating a disease in a host, comprising the following steps. (a) loading professional antigen-presenting cells with an antigen by contacting the cells with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) administering an effective amount of a composition comprising the loaded professional antigen-presenting cells to the host. In some embodiments, the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid.

[0020] In still another aspect, the invention provides a method of loading professional antigen-presenting cells, such as dendritic cells, with an antigen(s), comprising contacting the cells *in vitro* or *ex vivo* with a modified microbe expressing the antigen, under suitable conditions and for a time sufficient to load the antigen-presenting cells. In some embodiments, proliferation of the microbe is attenuated. In some embodiments, the microbe maintains sufficient gene expression to effect antigen presentation by the cell, even though proliferation of the microbe is attenuated. The antigen presentation can be MHC class I presentation or MHC class II presentation.

[0021] In another aspect, the invention provides a method of activating and/or maturing antigen-presenting cells (for instance, dendritic cells) comprising contacting the antigen-presenting cells *in vitro* or *ex vivo* with a modified microbe under suitable conditions and for a time sufficient to activate the dendritic cells and/or to allow the antigen-presenting cells to mature. In one embodiment, proliferation of the microbe is attenuated. In another embodiment, the microbe maintains sufficient gene expression to

effect activation and/or maturation of the cell, even though proliferation of the microbe is attenuated.

[0022] In still another aspect, the invention provides a method of inducing an immune response to an antigen, comprising administering to the host an effective amount of an immunogenic composition comprising an antigen presenting cell (such as a dendritic cell) presenting the antigen, wherein the antigen-presenting cell comprises a modified microbe. In one embodiment, proliferation of the microbe has been attenuated. In another embodiment, the microbe maintains sufficient gene expression to effect antigen presentation by the cell, even though proliferation of the microbe is attenuated. In one embodiment, the immune response is a CD8<sup>+</sup> T-cell response. In another embodiment, the immune response is a CD4<sup>+</sup> T-cell response.

[0023] In yet another aspect, the invention provides a method of inducing an immune response to an antigen, comprising the following steps: (a) contacting antigen-presenting cells (such as dendritic cells) *in vitro* or *ex vivo* with *Listeria* expressing the antigen under suitable conditions and for a time sufficient to load the antigen-presenting cells with the antigen and to effect activation and/or maturation of the antigen-presenting cells; and (b) administering an effective amount of the antigen-presenting cells to the host. In one embodiment, proliferation of the microbe is attenuated. In another embodiment, the microbe which is contacted with the antigen-presenting cells maintains sufficient gene expression to effect both presentation of the antigen on the antigen-presenting cell and activation and/or maturation of the antigen-presenting cell, even though proliferation of the microbe is attenuated. In one embodiment, the immune response is a CD8<sup>+</sup> T-cell response. In another embodiment, the immune response is a CD4<sup>+</sup> T-cell response.

[0024] In another aspect, the invention provides an *ex vivo* or *in vitro* professional antigen-presenting cell comprising a modified microbe, wherein proliferation of the microbe is attenuated. In another embodiment, the modified microbe maintains sufficient gene expression to effect antigen presentation by the dendritic cell, even though proliferation of the microbe is attenuated. In one embodiment, the antigen-presenting cell is a dendritic cell.

[0025] In yet another aspect, the invention provides a vaccine comprising an antigen-presenting cell (such as a dendritic cell), wherein the antigen-presenting cell comprises a modified microbe. In one embodiment, the microbe is *Listeria*. In one embodiment, proliferation of the *Listeria* has been attenuated. In another embodiment, the

*Listeria* maintains sufficient gene expression to effect antigen presentation on the cell, even though proliferation of the *Listeria* is attenuated.

[0026] In a still further aspect, the invention provides a pharmaceutical composition comprising an antigen-presenting cell (such as a dendritic cell) and a pharmaceutically acceptable carrier, wherein the antigen-presenting cell comprises modified *Listeria*. In one embodiment, proliferation of the *Listeria* has been attenuated. In another embodiment, the *Listeria* maintains sufficient gene expression to effect antigen presentation by the cell, even though proliferation of the *Listeria* is attenuated.

[0027] In some embodiments of each of the aforementioned aspects, the modified microbe is a modified *Listeria*. In additional embodiments of each of the aforementioned aspects, the *Listeria* is *Listeria monocytogenes*. In still further embodiments, the *Listeria* comprises a mutation in one or more genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD*, and *recA*. For instance, in any of the aforementioned aspects, the *Listeria* optionally comprises a mutation in *uvrAB*. In alternative embodiments, the *Listeria* optionally comprises both a mutation in *uvrAB* and *actA*.

[0028] In other embodiments of each of the aforementioned aspects, the attenuation of the *Listeria* has been effected by exposure of the *Listeria* to a cross-linking agent. In some embodiments of each of the aforementioned aspects, the cross-linking agent is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments of each of the aforementioned aspects, the cross-linking agent is a psoralen derivative and the *Listeria* is exposed to UVA light. In some embodiments of each of the aforementioned aspects, the cross-linking agent is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen (also referred to herein as "S-59").

## DRAWINGS

[0029] Figure 1 shows the attenuation of wild-type *Listeria* DP-L4056 containing OVA antigen as a function of psoralen S-59 concentration (2 J/cm<sup>2</sup> UVA) along with the measurement of OVA antigen presentation to a dendritic cell line. The bacterial log titer and % of antigen presented relative to untreated (data is for 100 *Listeria* per DC 2.4 cell) are plotted vs. nM S-59.

[0030] Figure 2 shows the attenuation of wild-type *Listeria* DP-L4056 (2A) and LLO- mutant DP-L4027 (2B) containing OVA antigen as a function of alkylator compound

I concentration along with the measurement of OVA antigen presentation to a dendritic cell line. The bacterial log titer and % of antigen presented relative to untreated (data is for 1 *Listeria* per DC 2.4 cell) are plotted vs.  $\mu\text{M}$  compound I.

[0031] Figure 3 shows a comparison of the inactivation of wild type *E. coli* to repair deficient mutant CSR 603 (*uvrA recA phr* mutant) as a function of S-59 concentration ( $2 \text{ J/cm}^2$  UVA). The bacterial log titer is plotted vs. nM S-59 (log scale).

[0032] Figure 4 shows the mean tumor volume as a function of days post implant of B16 OVA tumors into C57Bl/6 mice that are vaccinated at days 3, 7, and 14. The vaccines tested are with and without S-59 treatment.

[0033] Figure 5 shows the percent survival as a function of days post implant of B16 OVA tumors into C57Bl/6 mice that are vaccinated at days 3, 7, and 14. The vaccines tested are with and without S-59 treatment.

[0034] Figure 6 shows flow cytometry results showing a population of spleen cells that are TNF- $\alpha$  and IFN- $\gamma$  positive from mice vaccinated with wild type *Listeria* with and without OVA expression, with and without S-59 UVA treatment (PCT). Figure 6A shows the population of cells specific for LLO<sub>190-210</sub>. Figure 6B shows the population of cells specific for OVA.

[0035] Figure 7 shows ELISPOT results showing the number of IFN- $\gamma$  spot forming cells per  $2 \times 10^5$  spleen cells upon stimulation with either SL8, LLO<sub>190-201</sub>, or LLO<sub>296-304</sub>, from mice vaccinated with the indicated wild type *Listeria* strains with or without S-59 UVA treatment (PCT).

[0036] Figure 8 shows the attenuation of *Listeria* strains with and without deletion of *uvrAB*. The log titer is plotted vs. nM concentration of psoralen S-59 used ( $6 \text{ J/cm}^2$ ). Figure 8A, strains DP-L4017 (L461T LLO mutant) and wild type (DP-L4056). Figure 8B, strains DP-L4017 and DP-L4029 ( $\Delta actA$ ).

[0037] Figure 9 shows the attenuation of DP-L4029 ( $\Delta actA$ ) *Listeria* strain containing OVA antigen as a function of psoralen S-59 concentration along with the measurement of OVA antigen presentation to a dendritic cell line. The parent strain (in this case,  $\Delta actA$ ; 9A, 9C) is compared to the strain with a *uvrAB* deletion ( $\Delta uvrAB$ ; 9B, 9D). The bacterial log titer and % of antigen presentation relative to untreated are plotted vs. nM S-59. Figures 9A, 9B, dosed with  $0.5 \text{ J/cm}^2$  UVA, washed *Listeria* once, dosed again with  $5.5 \text{ J/cm}^2$  UVA, antigen presentation measured at 1 *Listeria* per DC 2.4 cell. Figures 9C, 9D, *Listeria* was grown in the presence of S-59, then dosed with  $6 \text{ J/cm}^2$  UVA,

antigen presentation measured at 10 *Listeria* per DC 2.4 cell. (Expanded plots of the data are also provided in Figures 9C and 9D.)

[0038] Figure 10 shows polyacrylamide gels of <sup>35</sup>S methionine/cysteine incorporated into protein synthesized by S-59/UVA treated *Listeria monocytogenes* strains DP-L4029 ( $\Delta actA$ ) and DP-L4029 *uvrAB* ( $\Delta actA \Delta uvrAB$ ).

[0039] Figure 11 shows the ELISPOT assay for spleen cells from mice vaccinated with S-59/UVA treated (two methods) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ )-OVA or  $\Delta actA \Delta uvrAB$ -OVA, stimulated with OVA specific antigen SL8, LLO specific antigens LLO 190 and LLO 296. Figure 11A shows spot forming colonies on plates stimulated with OVA specific antigen, Figure 11B plots the IFN- $\gamma$  spot forming cells per  $2 \times 10^5$  spleen cells for all three antigens.

[0040] Figure 12 shows the Intracellular Cytokine Staining (ICS) assay for spleen cells from mice vaccinated with S-59/UVA treated (two methods) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ )-OVA or  $\Delta actA \Delta uvrAB$ -OVA, stimulated with OVA derived T cell epitope SL8 (12A), LLO specific class II antigen LLO<sub>190-201</sub> (12B), or LLO specific class I antigen LLO<sub>296-304</sub> (12C). The S-59/UVA treated *Listeria* are marked "PCT" (stands for photochemical treatment) in the figure.

[0041] Figure 13 shows the number of colony forming units isolated per spleen (13A) or liver (13B) from mice vaccinated with S-59/UVA treated (two methods) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ ) or  $\Delta actA \Delta uvrAB$  and challenged with wild type *Listeria monocytogenes* thirty days after vaccination.

[0042] Figure 14 shows the number of colony forming units isolated per spleen (14A) or liver (14B) from mice vaccinated with S-59/UVA treated (grown with psoralen, then UVA treated) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ ) or DP-L4029  $\Delta actA \Delta uvrAB$  (1x, 3x, or 5x vaccination) and challenged with wild type *Listeria monocytogenes* thirty days after vaccination.

[0043] Figure 15 shows the antibody titer of *Listeria* specific antibodies from serum of mice vaccinated with S-59/UVA treated (grown with psoralen, then UVA treated) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ ) or  $\Delta actA \Delta uvrAB$  (1x, 3x, or 5x vaccination).

[0044] Figure 16 shows the percent survival (10 days post challenge) of mice vaccinated with S-59/UVA treated (grown with psoralen, then UVA treated) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ ) or  $\Delta actA \Delta uvrAB$  (1x, 3x, or 5x vaccination) and

challenged with 20 x LD<sub>50</sub> or 100 x LD<sub>50</sub> wild type *Listeria monocytogenes* thirty days after vaccination.

[0045] Figure 17 shows the results of an ICS assay for spleen cells from mice vaccinated with S-59/UVA treated (grown with psoralen, then UVA treated) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ )-OVA AH1A5 or  $\Delta actA \Delta uvrAB$ -OVA AH1A5, stimulated with antigens LLO91, AH1, AH1A5, or cells P815 or CT26 cells.

[0046] Figure 18 shows the results of an ELISPOT assay showing plates with spot forming colonies for spleen cells from mice vaccinated with S-59/UVA treated (grown with psoralen, then UVA treated) *Listeria monocytogenes* strains DP-L4029( $\Delta actA$ )-OVA AH1A5 or  $\Delta actA \Delta uvrAB$ -OVA AH1A5, stimulated with AH1A5 (18A) or AH1 (18B) antigen.

[0047] Figure 19 shows lungs from mice with established CT26 lung tumors given a therapeutic vaccination with S-59/UVA treated DP-L4029, with or without a  $\Delta uvrAB$  mutation (19A). The number of lung metastases are plotted for each vaccine strain (19B). The survival of the remaining mice is plotted in Figure 19C.

[0048] Figure 20 shows mice with established CT26 tumors were given therapeutic vaccination with *Listeria monocytogenes*  $\Delta actA$ ,  $\Delta actA$  AH1-A5,  $\Delta actA \Delta uvrAB$  AH1-A5 and  $\Delta actA \Delta inlB$  AH1-A5. The  $\Delta uvrAB$  strain was either no treatment, heat-killed (HK) or S-59 UVA (PCT) treated. The lungs harvested from a subset of the mice are shown in Figure 20A, with the number of lung metastases in each group plotted in Figure 20B. Survival of the remaining mice is plotted in Figure 20C (parent strain) and 20D ( $\Delta uvrAB$  strain).

[0049] Figure 21A shows fluorescent microscopy images of DC 2.4 cells infected by wild type *Listeria monocytogenes*  $uvrAB$  mutant that has been S-59/UVA treated, showing merged image (both *Listeria* and actin positive) and Rhodamine image (only actin positive). Figure 21B is a plot of the percentage of the *Listeria monocytogenes* that is in the cytoplasm for wild type and  $\Delta uvrAB$  strains (live, heat-killed or S-59 UVA treated) compared to LLO.

[0050] Figure 22 shows a negative image photomicrograph of Gram stained *Listeria monocytogenes* wild-type and  $\Delta uvrAB$  strains that have been S-59/UVA treated.

[0051] Figure 23 shows the target cell populations following injection into mice vaccinated with the indicated *Listeria* strains or vehicle control. The reduced levels of antigen-specific target cells relative to non-specific target cells indicates *in vivo*



cytotoxicity of T cells in response to the vaccination. Figure 23A shows results for AH1-A5 expressing vaccines with vaccination at days 0 (also 1 and 2 for S-59 UVA treated strains). (The top row in 23A and 23B shows results for mice vaccinated with the indicated vaccines for AH1 target cells. The bottom row shows results for mice vaccinated with the indicated vaccines for AH1-A5 target cells.) Figure 23B has a repeat vaccination at day 14 (15 and 16 for S-59 UVA treated) and Figure 23C looks at an OVA specific response.

[0052] Figure 24 shows the attenuation of *Bacillus anthracis* Sterne strain with and without deletion of *uvrAB*. The log titer is plotted vs. nM concentration of psoralen S-59 present during growth and UVA irradiation ( $6 \text{ J/cm}^2$ ).

[0053] Figure 25 shows *Listeria uvrAB* are more susceptible to S-59/UVA light inactivation. *Listeria* were grown to mid-log phase, washed in PBS, incubated for 5 min with varying concentrations of S-59 and illuminated at  $2.1 \text{ J/cm}^2$  of UVA light. The viability of *Listeria* was assessed by growth on BHI agar plates. (A) Representative BHI agar plates of *Listeria* treated at 100 nM S-59. Heat-killed *Listeria* served as control; (B) Viability of *Listeria* treated at varying concentrations of S-59 to form colonies on BHI agar plates.

[0054] Figure 26 shows that S-59/UVA treated, non-viable *Listeria uvrAB* retain their metabolic activity and the expression of their genomic repertoire. (A) Metabolic activity determined in a MTT assay of S-59/UVA inactivated *Listeria uvrAB*. Live and heat-killed *Listeria uvrAB* served as control; (B) Quantification of the metabolic activity of inactivated *Listeria uvrAB* strain determined in a MTT assay.

[0055] Figure 27 shows that fully inactivated *Listeria uvrAB* retain their capacity to infect DC and to escape from the phagolysosome. The murine DC line, DC2.4, grown on coverslips was infected at an MOI of 1 for 30 min at  $37^\circ\text{C}$ . Extracellular bacteria were carefully removed by several washes and infected cells were incubated for 5 hrs at  $37^\circ\text{C}$  in the presence of gentamicin to prevent growth of extracellular bacteria. DC2.4 cells were fixed with 3.5% formaldehyde and then stained with rabbit anti-*Listeria* antibody, detected with a goat-anti-rabbit FITC secondary antibody. Actin was detected with Phalloidin-rhodamine and the nucleus was visualized using DAPI.

[0056] Figure 28 shows that fully inactivated *Listeria uvrAB* efficiently load antigen into the MHC class I pathway of murine bone marrow-derived DC (BM-DC). Day 5 BM-DC were infected with a MOI of 100 for 30 min at  $37^\circ\text{C}$ . Extracellular bacteria were removed by several washes. Infected BM-DC were co-incubated with B3Z overnight and activation was determined by hydrolysis of the chromogenic substrate CPRG (absorbance).

[0057] Figure 29 shows that *Listeria* infected human immature monocyte-derived DC upregulate activation (29A) and maturation markers (29B) as well as secrete pro-inflammatory cytokines (29C). DCs were infected with *Listeria* at different MOI for 1 hour. Infected DCs were cultured for additional 24 hours in the presence of gentamicin to prevent the growth of extracellular bacteria. Phenotypic changes were determined by flow cytometry. Cytokine levels were determined from cell supernatants using the Cytometric bead array kit (Pharmingen).

[0058] Figure 30 shows that S-59/UVA inactivated *Listeria uvrAB* OVA induce OVA-specific immunity *in vivo*. Female C57BL/6 mice were administered intravenously with  $1 \times 10^8$  CFU of S-59/UVA inactivated *Listeria uvrAB* OVA. The S-59/UVA inactivated parent *Listeria* strain and heat-killed *Listeria* served as control. Seven days later, spleens were harvested and OVA-specific CD8+ T cell responses were assessed by IFN- $\gamma$  ELISPOT. (A) Representative ELISPOT wells are shown; (B) OVA-specific immunity assessed by ELISPOT. Spleen cells of vaccinated mice were cultured with or without OVA257-264 peptide.

[0059] Figure 31 shows the primary amino acid sequence of the heterologous antigen LLO-OVA/PR3 (SEQ ID NO:48). The figure also shows the OVA H-2 K<sup>b</sup> epitope (SEQ ID NO:49) and the PR3 HLA A-2 restricted class I epitope (a.k.a. PR1) (SEQ ID NO:50).

[0060] Figure 32 shows the compound 4'-(4-amino-2-oxa)butyl-4,5,8-trimethylpsoralen (S-59).

## DETAILED DESCRIPTION OF THE INVENTION

[0061] The present invention involves modified free-living microbes and the use of modified free-living microbes in vaccine compositions, wherein the nucleic acid of the microbe is modified so that proliferation of the microbe is attenuated. In some embodiments, the microbial gene expression is substantially unaffected by the modification. The present invention also involves the use of the modified microbes for antigen loading and induction of the activation/maturation of antigen presenting cells (APCs), *in vitro* or *ex vivo*. The antigen may be either an antigen produced naturally by the modified microbe, or may be a heterologous antigen expressed by a recombinant microbe. The resulting antigen presenting cells are suitable for use in vaccine compositions and for

immunotherapy. The immune response stimulated by administration of the resulting vaccine compositions may be a CD4<sup>+</sup> or a CD8<sup>+</sup> immune response.

[0062] One such modified microbe is *Listeria monocytogenes*. The inventors have engineered *Listeria* to be particularly sensitive to inactivation by psoralens, a group of compounds that form irreversible cross-links in the genomes of bacteria after illumination with ultraviolet A (UVA) light, so that they are non-viable. (See Example 3, below.) The attenuation of proliferation of wild-type and modified *Listeria* while maintaining expression of model antigens has now been shown (see Example 1-2 and 11, below). The modified *Listeria* is also shown to provide an anti-tumor response (Examples 4 and 14-16, below) and induce antigen-specific T-cell responses (Example 5) and *in vivo* cytotoxic responses (Example 20). *Listeria* is rapidly phagocytosed by DC and transported into the phagolysosomal compartment. This encounter results in the phenotypic maturation of the DC and subsequent secretion of a broad profile of immunostimulatory cytokines, including IFN- $\gamma$ , IL-12, and TNF- $\alpha$ . The inventors have now demonstrated that infection of immature DC with recombinant *Listeria* results in rapid DC activation/maturation, together with MHC class I-restricted presentation of an encoded heterologous antigen.

Additionally, degradation of *Listeria* vaccines within the phagolysosome results in presentation of encoded antigen via the MHC class II pathway. (See Examples, below)

[0063] Another such modified microbe is *Bacillus anthracis*. The inventors have also engineered attenuated strain of *Bacillus anthracis* which are particularly sensitive to inactivation by psoralens (see Example 21, below).

[0064] Accordingly, the invention provides a vaccine comprising a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. In some embodiments, the attenuation of the proliferation of the microbe is controllable in a dose-dependent manner. In some embodiments, microbial gene expression in the microbe is substantially unaffected by attenuation of the proliferation of the microbe. In some embodiments, the microbe in the vaccine expresses an antigen at a sufficient level to induce an immune response to the antigen in an individual upon administration of the vaccine to the individual. In some embodiments, the nucleic acid has been modified by reaction with a nucleic acid targeted compound which reacts directly with the nucleic acid. In one embodiment, the nucleic acid target compound is an alkylator such as  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., 4'-

(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, also referred to herein as "S-59") activated by UVA irradiation. In some embodiments, the microbe in the vaccine comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified. In some embodiments, the microbe is a bacterium, such as *Bacillus anthracis* or *Listeria monocytogenes*. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. In some embodiments, the vaccine further comprises a pharmaceutically acceptable carrier and/or an adjuvant. The invention further provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the vaccine. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the vaccine, wherein the microbe expresses the antigen.

[0065] The invention also provides an isolated mutant *Listeria* strain, such as a mutant *Listeria monocytogenes* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the mutant *Listeria* strain is defective with respect to at least one DNA repair enzyme (such as UvrA and/or UvrB). In some embodiments, the mutant *Listeria* strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the mutant strain is the *Listeria monocytogenes*  $\Delta actA/\Delta uvrAB$  strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563. In other embodiments, the strain is a mutant of the *Listeria monocytogenes*  $\Delta actA/\Delta uvrAB$  strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563, wherein the mutant of the deposited strain is defective with respect to UvrA, UvrB, and ActA. The invention further provides vaccines and professional antigen-presenting cells comprising the mutant *Listeria* strain. Methods of using the modified *Listeria* strain to induce immune responses and to prevent or treat disease are also provided.

[0066] The invention provides an isolated mutant *Bacillus anthracis* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the mutant strain is defective with respect to at least one DNA repair enzyme (such as UvrA and/or UvrB). In some embodiments, the mutant strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the mutant strain is attenuated with respect to RecA. In some embodiments, the mutant strain comprises a genetic mutation in the *recA* gene. In some embodiments, the mutant strain comprises one or more mutations in the *lef* gene, *cya* gene, or both genes, that decreases the toxicity of the

strain. The invention further provides vaccines and professional antigen-presenting cells comprising the mutant strain. Methods of using the modified *Bacillus anthracis* strain to induce immune responses and to prevent or treat disease are also provided.

[0067] In addition, the invention provides a professional antigen-presenting cell (e.g., a dendritic cell) comprising a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. In some embodiments, the attenuation of the proliferation of the microbe is controllable in a dose-dependent manner. In some embodiments, microbial gene expression in the microbe is substantially unaffected by attenuation of the proliferation of the microbe. In some embodiments, the microbe in the vaccine expresses an antigen at a sufficient level to induce an immune response to the antigen in an individual upon administration of the vaccine to the individual. In some embodiments, the nucleic acid has been modified by reaction with a nucleic acid-targeted compound which reacts directly with the nucleic acid. In one embodiment, the nucleic acid target compound is an alkylator such as  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments, the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation. In some embodiments, the microbe in the vaccine comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified. In some embodiments, the microbe is a bacterium. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. The invention also provides a vaccine comprising the antigen-presenting cell. The invention further provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the antigen-presenting cell. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the antigen-presenting cell, wherein the microbe expresses the antigen. The invention further provides a method of activating naïve T cells *ex vivo* or *in vitro*, comprising contacting the naïve T cells with the professional antigen-presenting cell under suitable conditions and for a sufficient time to activate the naïve T-cells.

[0068] The invention provides a method of loading professional antigen-presenting cells with an antigen comprising contacting the professional antigen-presenting cells with a free-living microbe that comprises a nucleic acid sequence encoding the antigen, under suitable conditions and for a sufficient time to load the professional antigen-presenting

cells, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation.

[0069] The invention also provides a method of activating and/or maturing professional antigen-presenting cells comprising contacting the professional antigen-presenting cells with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, under suitable conditions and for a sufficient time to load the professional antigen-presenting cells, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation.

[0070] The invention further provides a method of preventing or treating a disease in a host, comprising the following steps. (a) loading professional antigen-presenting cells with an antigen by contacting the cells with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) administering an effective amount of a composition comprising the loaded professional antigen-presenting cells to the host.

[0071] The invention also provides a method of loading antigen-presenting cells, such as dendritic cells, with an antigen, comprising contacting the cells *in vitro* or *ex vivo* with a modified microbe expressing the antigen, under suitable conditions and for a time sufficient to load the antigen-presenting cells.

[0072] The invention provides a method of activating and/or maturing antigen-presenting cells comprising contacting the antigen-presenting cells *in vitro* or *ex vivo* with a modified microbe under suitable conditions and for a time sufficient to effect activation and/or maturation of the dendritic cells and/or to allow the antigen-presenting cells to mature.

[0073] The invention provides a method of inducing an immune response to an antigen, comprising administering to the host an effective amount of an immunogenic composition comprising an antigen presenting cell presenting the antigen, wherein the antigen-presenting cell comprises a modified microbe.

[0074] In addition, the invention provides a method of inducing an immune response to an antigen, comprising the following steps: (a) contacting antigen-presenting cells *in vitro* or *ex vivo* with *Listeria* expressing the antigen under suitable conditions and for a time sufficient to load the antigen-presenting cells with the antigen and to effect activation and/or maturation of the antigen-presenting cells; and (b) administering an effective amount of the antigen-presenting cells to the host. In one embodiment, proliferation of the microbe is attenuated.

[0075] The invention also provides an *ex vivo* or *in vitro* professional antigen-presenting cell comprising a modified micro be, wherein proliferation of the microbe is attenuated.

[0076] Additionally, the invention provides a vaccine comprising an antigen-presenting cell, wherein the antigen-presenting cell comprises a modified microbe and a pharmaceutical composition comprising an antigen-presenting cell and a pharmaceutically acceptable carrier, wherein the antigen-presenting cell comprises *Listeria*.

#### Microbe-Based Vaccines

[0077] The present invention involves modified free-living microbes and the use of modified free-living microbes in a vaccine composition, wherein the nucleic acid of the microbe is modified so that proliferation of the microbe is attenuated. In some embodiments, the microbial gene expression is substantially unaffected by the modification.

[0078] It has been observed that killed microbial vaccines are often inferior to live attenuated microbial vaccines [Lauvau et al., Science 294:1735-1739 (2001)]. In completely killed microbes, the *de novo* microbial gene expression is essentially eliminated. Therefore, the modification of the microbial nucleic acid to an appropriate level such that proliferation is attenuated while maintaining a sufficient level of microbial gene expression may be more effective than a killed microbial vaccine and provides an approach to vaccine preparation that can be applied to any microbial vector, whether the vaccine targets the prevention of infectious disease caused by the microbial vector, or the vector is used to deliver a heterologous antigen. It is to be understood that the use of the term microbes as it relates to all embodiments of the present invention is intended to mean free-living microbes and is not intended to include viruses. Such a microbe-based vaccine may be used to deliver a specific antigen to an individual. In one embodiment, the vaccine delivers more than one antigen. Such vaccines are designed to stimulate an immune response to one or more antigens, resulting in an individual who is immunized against the antigen or antigens. The immune response that is generated can be either an antibody mediated response, a cell mediated response, or both. The term vaccine is intended to encompass a preventative vaccine, i.e. one that is given to stimulate an immune response so that if the individual subsequently is exposed to the antigen in nature, the pre-formed immune response will increase the individual's ability to fight off the agent or cells carrying the antigen. The term vaccine is also intended to encompass a therapeutic vaccine, i.e. one

that is given to an individual who already has a disease associated with the vaccine antigen, wherein the vaccine can elicit an immune response or boost the individual's existing immune response to the antigen to provide an increased ability to fight the agent or cells carrying the antigen. This includes an immune response to a diseased cell, such as a cancer cell, as well as an immune response to a disease associated protein such as a prion. In one embodiment, the free-living microbe is selected from the group consisting of bacteria, protozoa, and fungi. In one embodiment, the free-living microbe is a bacteria selected from the group consisting of Gram positive bacteria, Gram negative bacteria, intracellular bacteria and mycobacteria.

The present invention includes various levels of modification of the nucleic acid of microbes. It is understood that the metabolism of the microbial nucleic acid occurs in several ways. Replication of the microbe involves the copying of the DNA of the entire microbial genome in order to replicate the microbe and the subsequent partitioning of the DNA molecules into separate cells, i.e. the cell divides with the resulting cells both having a complete copy of the DNA of the microbial genome. Microbial nucleic acid metabolism also involves the combination of transcription of DNA into RNA and translation of RNA to produce proteins. The transcription of the microbial genome involves the copying of portions of the DNA of the microbial genome into RNA, either messenger or transfer RNA. The translation of the messenger RNA involves the reading of this RNA in order to produce a specific protein or portion of a protein. In the present invention the nucleic acid of a population of microbes is modified to a desired extent based upon the nature of the microbe and its intended use. In some embodiments, the desired extent of modification is such that replication of the microbe's genome is significantly attenuated while the production of proteins remains sufficiently active (i.e. the microbe is metabolically active). It is to be understood that whatever the nature of the modification, the level of modification can be represented in terms of the number of modifications on average per base pair of the microbial genome. For example, if the modification is due to covalent binding of a compound to the nucleic acid (adducts), the modification can be represented in terms of the average number of base pairs between adducts. The microbes of the invention can be modified to levels of about 1 modification per  $10^4$ - $10^8$  base pairs, also about 1 modification per  $10^4$ - $10^7$ , also about 1 modification per  $10^5$ - $10^7$ , or about 1 modification per  $10^5$ - $10^6$  base pairs. In one embodiment, the level of modification is adjusted to the minimum amount required to block DNA replication in the microbial population, such that the population shows no observable proliferation, while maintaining sufficient activity of transcription and



translation of individual genes (i.e. maintains some metabolic activity) to achieve a safe and effective vaccine.

[0079] In one aspect, the invention provides a vaccine comprising a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. In some embodiments, the attenuation of the proliferation of the microbe is controllable in a dose-dependent manner. In some embodiments, microbial gene expression in the microbe is substantially unaffected by attenuation of the proliferation of the microbe. In some embodiments, the microbe in the vaccine expresses an antigen at a sufficient level to induce an immune response to the antigen in an individual upon administration of the vaccine to the individual. In some embodiments, the nucleic acid has been modified by reaction with a nucleic acid targeted compound which reacts directly with the nucleic acid. In one embodiment, the nucleic acid target compound is an alkylator such as  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, also referred to herein as "S-59") activated by UVA irradiation. In some embodiments, the microbe in the vaccine comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified. In some embodiments, the microbe is a bacterium, such as *Bacillus anthracis* or *Listeria monocytogenes*. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. In some embodiments, the vaccine further comprises a pharmaceutically acceptable carrier and/or an adjuvant. The invention further provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the vaccine. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the vaccine, wherein the microbe expresses the antigen.

[0080] The invention further provides vaccines comprising a mutant *Listeria monocytogenes* strain or a mutant *Bacillus anthracis* strain, wherein the mutant *Listeria monocytogenes* strain or *Bacillus anthracis* strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid.

#### Antigen-presenting cell vaccines.

[0081] The present invention involves modified free-living microbes and the use of modified free-living microbes in the preparation of vaccine compositions based on antigen-

presenting cells, wherein the nucleic acid of the microbe is modified so that proliferation of the microbe is attenuated. In some embodiments, the microbial gene expression of the modified microbe is substantially unaffected.

[0082] In one embodiment of the invention, the antigen-presenting cells used in the vaccines are professional antigen presenting cells. Professional antigen-presenting cells include macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. In one embodiment, the professional antigen-presenting cells are dendritic cells. In another embodiment, the professional antigen-presenting cells are macrophages or dendritic cells (DCs). In one embodiment the antigen-presenting cells are human cells.

[0083] In one embodiment, immature antigen-presenting cells, such as DCs, are isolated from a patient and infected with a modified microbe expressing an antigen. The resulting, loaded, antigen-presenting cells are then transferred back into the patient as an autologous APC vaccine, thereby inducing either a CD4+ or a CD8+ immune response.

[0084] Accordingly, one example of a method of preparing and using an antigen-presenting cell vaccine of the invention is as follows: Immature DCs are isolated from colon cancer patients and infected with S-59/UVA-inactivated, non-viable, metabolically active recombinant *Listeria*-CEA vaccines. DC infection with *Listeria* results in efficient loading of CEA tumor antigen into the MHC class I and II pathways. *Listeria* infection stimulates DC to undergo rapid activation and maturation, critical for DC to become potent APCs capable of inducing primary T cell responses *in vivo*. Mature DC upregulate the expression of CD83, co-stimulatory molecules such as CD80, CD86, as well as MHC molecules. *Listeria* vaccine-loaded DCs are washed and infused back into the patient as an autologous DC vaccine to stimulate a CEA-specific T cell response.

[0085] Particular embodiments are exemplified in the specific Examples listed below. It is understood, however, that the general methods and techniques described herein may be more broadly applied to a wide variety of modified microbes, antigens, and diseases. One of ordinary skill in the art will be able to readily adapt the teachings described herein.

[0086] In an alternative embodiment, immature antigen-presenting cells, such as DCs, are infected *in vitro* with a modified microbe expressing an antigen. The resulting, loaded, antigen-presenting cells are then used to prime a T-cell population which is then

transferred into the patient, thereby inducing either a CD4+ or a CD8+ immune response to the antigen.

[0087] In another aspect, the invention provides a professional antigen-presenting cell (e.g., a dendritic cell) comprising a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. In some embodiments, the attenuation of the proliferation of the microbe is controllable in a dose-dependent manner. In some embodiments, microbial gene expression in the microbe is substantially unaffected by attenuation of the proliferation of the microbe. In some embodiments, the microbe in the vaccine expresses an antigen at a sufficient level to induce an immune response to the antigen in an individual upon administration of the vaccine to the individual. In some embodiments, the nucleic acid has been modified by reaction with a nucleic acid targeted compound which reacts directly with the nucleic acid. In one embodiment, the nucleic acid target compound is an alkylator such as  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other embodiments, the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation. In some embodiments, the microbe in the vaccine comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified. In some embodiments, the microbe is a bacterium. In some embodiments, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. The invention also provides a vaccine comprising the antigen-presenting cell. The invention further provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the antigen-presenting cell. The invention also provides a method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the antigen-presenting cell, wherein the microbe expresses the antigen. The invention further provides a method of activating naïve T cells *ex vivo* or *in vitro*, comprising contacting the naïve T cells with the professional antigen-presenting cell under suitable conditions and for a sufficient time to activate the naïve T-cells.

#### Attenuation of microbial replication

[0088] The present invention involves the modification of microbial nucleic acid in order to attenuate replication of the microbe. This attenuation in replication can be used to increase the level of safety upon administration of the microbes to individuals. The ability of a microbe to proliferate can be measured by culturing a population of microbes under

conditions that provide normal growth. The normal growth of a population of microbes is considered to be the growth of microbes having no modifications to the nucleic acid of the microbe. The modification of the microbial genome will result in some attenuation so that the microbe will not undergo normal growth. Some microbes will form colonies that can be counted on solidified growth medium. Attenuation of the replication of the microbe can thus be measured as a reduction in the number of colony forming units (CFU). A stock solution of the microbe colony will be serially diluted until the number of colony forming units can be easily measured (e.g. 50-500 CFU). Typically, dilutions are 10-fold and the number of colonies counted for one or more of the diluted samples is used to estimate the log titer of the sample. For example, an aliquot of diluted microbe stock is plated on growth media and the resulting colonies are counted. The colony forming units per mL (CFU/mL) of the dilution is calculated, and the colony forming units per mL of the original stock (known as the titer) is calculated from the dilution. The log number is known as the log titer. As an example, 24 colony forming units on plating a 0.2 mL aliquot of a  $1 \times 10^5$  dilution gives a  $1.2 \times 10^7$  titer, or 7.08 log titer stock. The attenuation can be measured as the comparison of microbial titer prior to modification of the microbial nucleic acid to that after modification of the microbial nucleic acid. The log of the ratio of the titer of unmodified microbe to the titer of microbe after modification represents the log attenuation (or simply the difference in log titer of the two). For example, if an unmodified microbe titer measures  $1.2 \times 10^7$  and a modified microbe titer measures  $4.3 \times 10^2$ , the resulting level of attenuation is 4.45 log. This method can be used to assess the attenuation of any microbe, whether pathogenic or non-pathogenic. For some microbes, rather than measuring the growth of the microbe directly, a plaque assay that measures the microbe by its ability to kill infected cells can be used. For example, certain intracellular bacteria can be grown on a lawn of mammalian cells that it can infect. After appropriate incubation conditions, the lawn can be observed for plaques (clear areas in the cell layer that represent killed cells). The above calculations are similar, where the number of plaque forming units is substituted for colony forming units to assess attenuation of the number of plaque forming units by modification of the nucleic acid of the microbe. For embodiments of the invention, the desired amount of attenuation can range from a two-fold reduction to much greater levels of attenuation, including a level where essentially no proliferation is observed, depending on the desired level of safety and the intended application of the microbe. A two-fold attenuation in replication would be observed if for a given dilution, there are half as many colonies (or plaques) in the population of a microbe where the nucleic acid is modified as

there are in an unmodified population of the microbe (about 0.3 log attenuation). In some embodiments, the attenuation is at least about 0.3 log, about 1 log, about 2 log, about 3 log, about 4 log, about 5 log, about 6 log, or at least about 8 log. In some embodiments, the attenuation is in the range of about 0.3 to > 10 log, about 2 to >10 log; about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, also about 0.3-7 log, also about 0.3-6 log, also about 0.3-5 log, also about 0.3-4 log, also about 0.3-3 log, also about 0.3-2 log, also about 0.3-1 log. In some embodiments, the attenuation is in the range of about 1 to >10 log, 1-8 log, 1-6 log, also about 2-6 log, also about 2-5 log, also about 3-5 log. In one embodiment of the invention, the attenuation results in essentially complete inactivation (e.g. where no colonies or plaques are observed to the limit of detection), wherein the microbial gene expression is sufficiently active. Such a population of microbes can be achieved by titrating the concentration of the agent used to modify the microbial nucleic acid to find the lowest concentration at which no colonies or plaques are observed at the limit of detection.

[0089] In the case of pathogenic microbes, it is also possible to assess the attenuation in terms of biological effects of the microbe. For example, the pathogenicity of a microbe can be assessed by measurement of the median lethality ( $LD_{50}$ ) in mice or other vertebrates. The  $LD_{50}$  is the amount (e.g. CFU) of microbe injected into the vertebrate that would result in the death of half of the population of the vertebrate. The  $LD_{50}$  values can be compared for modified and unmodified microbes as a measure of the amount of attenuation. For example, if an unmodified population of microbes has an  $LD_{50}$  of  $10^3$  microbes and the population of microbes in which the nucleic acid has been modified has an  $LD_{50}$  of  $10^5$  microbes, the microbe has been attenuated so that its  $LD_{50}$  is increased 100-fold, or by 2 log. In some embodiments, the  $LD_{50}$  is 2-fold to 1000-fold higher. In some embodiments, an attenuated strain is used that already has a relatively high  $LD_{50}$ . In such cases, the modified microbes increase in  $LD_{50}$  will be limited by how much material can be infused without causing harm. For example, the  $LD_{50}$  of a heat killed organism would not be much higher than about  $1-5 \times 10^9$  simply because of the loading of biological material into the mice and/or the inflammatory reaction to the bacterial wall components. The degree of attenuation may also be measured qualitatively by other biological effects, such as the extent of tissue pathology or serum liver enzyme levels. Typically, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and bilirubin levels in the serum are determined at a clinical laboratory for mice injected with microbes of the present invention. Comparisons of these effects in mice or other vertebrates would be made for unmodified and modified microbe as a way to assess the attenuation of the

microbe. In addition to measuring the effects of the microbes on the tissues, the amount of viable microbe that can be recovered from infected tissues such as liver or spleen as a function of time could also be used as a measure of attenuation by comparing these values in mice injected with unmodified vs. modified microbes.

Expression of proteins by microbes of the invention.

[0090] The modification of the nucleic acid of the microbe, in addition to attenuating proliferation of the microbe, is controlled so that microbial gene expression is substantially unaffected. To be substantially unaffected, the microbial gene expression need not be completely active upon modification of the nucleic acid. It is only necessary that in a population of a microbe in which the nucleic acid is modified to attenuate replication, microbial gene expression is sufficiently active to provide an adequate level of expression of the desired protein by the microbe. An adequate level of expression depends to some extent on the intended use of the microbe. For example, if the microbe contains a particular antigen that is to be used as a vaccine, adequate expression would be determined as the minimum level of expression that provides an effective protective or therapeutic immune response to the vaccine. The microbial gene expression can also be assessed by both *in vitro* and *in vivo* methods in order to assess whether such a vaccine might provide an effective immune response. In general, a population of a microbe in which the nucleic acid has been modified can be compared to an unmodified population of the microbe with respect to a particular antigen.

[0091] One possibility is to measure the presentation of the antigen of interest by an antigen presenting cell that has been mixed with a population of the microbe. The microbes may be mixed with a suitable antigen presenting cell or cell line, for example a dendritic cell, and the antigen presentation by the dendritic cell to a T cell that recognizes the antigen can be measured. If the microbes are expressing the antigen at a sufficient level, it will be processed into peptide fragments by the dendritic cells and presented in the context of MHC class I or class II to CD8+ or CD4+ T cells, respectively. For the purpose of detecting the presented antigen, a T cell clone or T cell line responsive to the particular antigen may be used. The T cell may also be a T cell hybridoma, where the T cell is immortalized by fusion with a cancer cell line. Such T cell hybridomas, T cell clones, or T cell lines can comprise either CD8+ or CD4+ T cells. The antigen presenting cell can present to either CD8+ or CD4+ T cells, depending on the pathway by which the antigens are processed. CD8+ T cells recognize antigens in the context of MHC class I while CD4+ T cells recognize antigens in the context of MHC class II. The T cell will be stimulated by

the presented antigen through specific recognition by its T cell receptor, resulting in the production of certain proteins, such as IL-2 or interferon- $\gamma$  (IFN- $\gamma$ ), that can be quantitatively measured (for example using an ELISA assay). Alternatively, a hybridoma can be designed to include a reporter gene, such as  $\beta$ -galactosidase, that is activated upon stimulation of the T cell hybridoma by the presented antigens. The increase in the production of  $\beta$ -galactosidase can be readily measured by its activity on a substrate, such as chlorophenolred- $\beta$ -D-galactopyranoside, which results in a color change. The color change can be directly measured as an indicator of specific antigen presentation (Examples 1, 2 and 11). Additional *in vitro* and *in vivo* methods for assessing the antigen expression of microbial vaccines of the present invention can be found in Example 5. It is also possible to directly measure the expression of a particular protein by microbes of the present invention. For example, a radioactively labeled amino acid can be added to a cell population and the amount of radioactivity incorporated into a particular protein can be determined. The proteins synthesized by the cell population can be isolated, for example by gel electrophoresis or capillary electrophoresis, identified as the protein of interest, e.g. by binding with an antibody-specific for the protein, and the amount of radioactivity can be quantitatively measured to assess the expression level of the particular protein. Alternatively, the proteins can be expressed without radioactivity and detected by various methods, such as an ELISA assay or by gel electrophoresis and Western blot with detection using an enzyme linked antibody or fluorescently labeled antibody.

[0092] While it is possible that the modification of the microbial nucleic acid reduces the level of protein expression as compared to an unmodified microbe, it is to be understood that this may still provide an effective vaccine. It is the combination of attenuation of proliferation with adequate protein expression that is important in some embodiments of the invention. The efficacy of a vaccine is generally related to the dose of antigen that can be delivered by the microbe, and in some instances, some level of active gene expression by the microbe is necessary. The attenuation of replication of the microbe may be several log while the microbial gene expression is still sufficiently maintained. If the same dose of an attenuated microbe is compared to that of an unmodified microbe, the resulting antigen expression (as assessed by the methods discussed above) in the attenuated microbe population is at least about 1%, about 5%, about 10%, about 25%, about 50%, about 75% or at least about 90% of the antigen expression in the unmodified microbe population. Since there may be several log attenuation in replication, the dose of the

modified microbe may be safely increased by up to several log, resulting in an equivalent or greater amount of the antigen presented by the attenuated microbes relative to unmodified microbes upon vaccination.

[0093] In some embodiments, a heterologous nucleic acid sequence encoding a protein may be codon-optimized to match the codon preference of the bacterial host expressing the protein. In addition, the sequence encoding a signal peptide fused to the expressed protein may also be codon-optimized to match the codon preference of the bacterial host. In preferred embodiments, the bacterial host is *Listeria* and either or both of the heterologous protein encoding sequence and the sequence encoding a signal peptide may be codon-optimized. For further information on codon optimization of antigens and signal sequences in *Listeria*, see U.S. application Serial No. 60/532,598, incorporated by reference herein.

#### Microbial nucleic acid modification

[0094] The nucleic acid of a population of a microbe can be modified by a variety of methods. The nucleic acid of the microbe can be modified by physical means, e.g. irradiation with ultraviolet light or ionizing radiation. Ionizing radiation, such as x-rays or  $\gamma$ -rays, may be used to cause single-strand or double-strand breaks in the nucleic acid. Ultraviolet radiation may be used to cause pyrimidine dimers in the nucleic acid. The appropriate dose of radiation is determined by assessing the effects of the radiation on replication and protein expression as detailed above.

[0095] The nucleic acid of the microbe can also be modified by chemical means, e.g. by reaction with a nucleic acid targeted compound. In one embodiment, the microbe is treated with a nucleic acid targeted compound that can modify the nucleic acid such that the proliferation of the microbe is attenuated, wherein the microbial population is still able to express a desired protein antigen to a degree sufficient to elicit an immune response. The nucleic acid targeted compound is not limited to a particular mechanism of modifying the nucleic acid. Such compounds modify the nucleic acid either by reacting directly with the nucleic acid (i.e. all or some portion of the compound covalently binds to the nucleic acid), or by indirectly causing the modification of the nucleic acid (e.g. by causing oxygen damage via generation of singlet oxygen or oxygen radicals, by generating radicals of the compound that cause damage, or by other mechanisms of reduction or oxidation of the nucleic acid). Eneidyne is an example of a class of compounds that form radical species that result in the cleavage of DNA double strands [Nicolau et al., Proc. Natl. Acad. Sci.



USA, 90:5881-5888 (1993)]. Compounds that react directly with the nucleic acid may react upon activation of the compound, for example upon radiation of the compound. Compounds that react indirectly to cause modification of the nucleic acid may require similar activation to generate either an activated species of the compound or to generate some other active species. While not being limited to the means for activation of nucleic acid targeted compounds, one embodiment of the invention includes the use of photoactivated compounds that either react directly with the nucleic acid or that generate a reactive species such as a reactive oxygen species (e.g. singlet oxygen) which then reacts with the nucleic acid.

[0096] The nucleic acid targeted compounds preferentially modify nucleic acids without significantly modifying other components of a biological sample. Such compounds provide adequate modification of the nucleic acid without significantly altering or damaging cell membranes, proteins, and lipids. Such compounds may modify these other cell components to some degree that is not significant. These cell components such as cell membranes, proteins and lipids are not significantly altered if their biological function is sufficiently maintained. In the case of treating a microbe with a nucleic acid targeted compound, the nucleic acid modification is such that the replication of the microbe is attenuated while the cell membranes, proteins and lipids of the microbe are essentially unaffected such that microbial gene expression is active (e.g. the enzymes required for this are not significantly affected), and the surface of the microbe maintains essentially the same antigenicity as a microbe that has not been treated with the compound. As a result, such compounds are useful in preparing an inactivated microbe for use as a vaccine since the proliferation of the microbe is sufficiently attenuated while maintaining sufficient antigenicity or immunogenicity to be useful as a vaccine. Because the compounds specifically modify nucleic acids, the modification can be controlled to a desired level so that replication is attenuated while maintaining a sufficient level of protein expression. The modification can be controlled by varying the parameters of the reaction, such as compound concentration, reaction media, controlling compound activation factors such as light dose or pH, or controlling compounds that cause oxygen damage by controlling the oxygen concentration (either physically, e.g. by degassing, or chemically, by use of oxygen scavengers). A nucleic acid targeted compound is any compound that has a tendency to preferentially bind nucleic acid, i.e. has a measurable affinity for nucleic acid. Such compounds have a stronger affinity for nucleic acids than for most other components of a biological sample, especially components such as proteins, enzymes, lipids and

membranes. The nucleic acid targeting provides specificity for the modification of nucleic acids without significantly affecting other components of the biological sample, such as the machinery for gene transcription and protein translation.

[0097] Compounds can be targeted to nucleic acids in a number of modes.

Compounds which bind by any of the following modes or combinations of them are considered nucleic acid targeted compounds. Intercalation, minor groove binding, major groove binding, electrostatic binding (e.g. phosphate backbone binding), and sequence-specific binding (via sequence recognition in the major or minor groove) are all non-covalent modes of binding to nucleic acids. Compounds that include one or more of these modes of binding will have a high affinity for nucleic acids. While the invention is not limited to the following compounds, some examples of compounds having these modes of binding to nucleic acid are as follows: intercalators are exemplified by acridines, acridones, proflavin, acriflavine, actinomycins, anthracyclines, beta-rhodomyacin A, daunomycin, thiaxanthones, miracil D, anthramycin, mitomycin, echinomycin, quinomycin, triostin, diacridines, ellipticine (including dimers, trimers and analogs), norphilin A, fluorenes and flourenones, fluorenodiamines, quinacrine, benzacridines, phenazines, phenanthradines, phenothiazines, chlorpromazine, phenoxazines, benzothiazoles, xanthenes and thioxanthenes, anthraquinones, anthrapyrazoles, benzothioapyranolindoles, 3,4-benzpyrene, benzopyrene diol epoxide, 1-pyrenyloxirane, benzanthracene-5,6-oxide, benzodipyrones, benzothiazoles, quinolones, chloroquine, quinine, phenylquinoline carboxamides, furocoumarins (e.g. psoralens, isopsoralens, and sulfur analogs thereof), ethidium salts, propidium, coralyne, ellipticine cation and derivatives, polycyclic hydrocarbons and their oxirane derivatives, and echinomycin; minor groove binders are exemplified by distamycin, mitomycin, netropsin, other lexitropsins, Hoechst 33258 and other Hoechst dyes, DAPI (4',6'-diamidine-2-phenylindole), berenil, and triarylmethane dyes; major groove binders are exemplified by aflatoxins; electrostatic binders are exemplified by spermine, spermidine, and other polyamines; and sequence-specific binders are exemplified by nucleic acids or analogues which bind by such sequence-specific interactions as triple helix formation, D-loop formation, and direct base pairing to single stranded targets. Other sequence-specific binding compounds include polypyrrole compounds, polypyrrole imidazole compounds, cyclopropylpyrroloindole compounds and related minor groove binding compounds [Wemmer, *Nature Structural Biology*, 5(3):169-171 (1998), Wurtz et al., *Chemistry & Biology* 7(3):153-161 (2000), Anthony et al., *Am. J. Pharmacogenomics* 1(1):67-81 (2001)].

[0098] In addition to targeting nucleic acids, the compounds are also able to react with the nucleic acid, resulting in covalent binding to the nucleic acid. Nucleic acid alkylators are a class of compounds that can react covalently with nucleic acid and include, but are not limited to, mustards (e.g. mono or bis haloethylamine groups, and mono haloethylsulfide groups), mustard equivalents (e.g. epoxides, alpha-halo ketones) and mustard intermediates (e.g. aziridines, aziridiniums and their sulfur analogs), methanesulphonate esters, and nitroso ureas. The nucleic acid alkylators typically react with a nucleophilic group on the nucleic acid. It is the combination of the nucleic acid alkylating activity and the nucleic acid targeting ability of these compounds that gives them the ability to covalently react specifically with nucleic acids, providing the desired modification of the nucleic acid of microbes for use in the present invention. The specificity of these compounds may be further enhanced by the use of a quencher that will not enter the microbe. Such a quencher will quench reactions with the surface of the microbe while still allowing the nucleic acid targeted compounds to react with the microbial nucleic acid. A discussion of such quenching can be found in US Patent number 6,270,952, the disclosure of which is hereby incorporated by reference. The modification of the microbial nucleic acid can be controlled by adjusting the compound concentration and reaction conditions. The appropriate concentration and reaction conditions are determined by assessing their effects on replication and protein expression as detailed above. The compounds used in the present invention are effective at concentrations of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1 nM to 10 µM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM. A discussion of nucleic acid targeted, nucleic acid reactive compounds for specific reaction with nucleic acids, in particular microbial nucleic acids, can be found in US patents 6,143,490 and 6,093,725, the disclosures of which are hereby incorporated by reference.

[0099] The nucleic acid can be modified by using a nucleic acid targeted compound that requires activation with radiation in order to cause the nucleic acid modification. Such compounds are targeted to nucleic acids as discussed above. These compounds include, but are not limited to, acridines, acridones, anthryl derivatives, alloxazines (e.g. riboflavin), benzotriazole derivatives, planar aromatic diazo derivatives, planar aromatic cyano derivatives, toluidines, flavines, phenothiazines (e.g. methylene blue), furocoumarins, angelicins, psoralens, sulfur analogs of psoralens, quinolones, quinolines, quinoxalines, naphthyridines, fluoroquinolones, anthraquinones, and anthracenes. Many of these

compounds are used as DNA photocleavage agents [Da Ros et al., Current Pharmaceutical Design 7:1781 (2001)]. While the invention is not limited to the method of activation of the nucleic acid targeted compounds, typically, the compounds can be activated with light of particular wavelengths. The effective wavelength of light depends on the nature of the compound and can range anywhere from approximately 200 to 1200 nm. For some of these compounds, activation causes modification of the nucleic acid without direct binding of the compound to the nucleic acid, for example by generating reactive oxygen species in the vicinity of the nucleic acid. For some of these compounds, activation results in binding of the compound directly to the nucleic acid (i.e. the compound binds covalently). Some of these compounds can react with the nucleic acid to form an interstrand crosslink. Psoralens are an example of a class of compounds that crosslink nucleic acids. These compounds are typically activated with UVA light (320-400 nm). Psoralen compounds for use in the present invention are exemplified in US patents 6,133,460 and 5,593,823, the disclosures of which are hereby incorporated by reference. Again, it is the combination of nucleic acid targeting and the ability to modify the nucleic acid upon activation that provide specific reactivity with nucleic acids. The modification of the microbial nucleic acid can be controlled by adjusting the compound concentration, reaction conditions and light dose. The appropriate concentration and light dose are determined by assessing their effects on replication and protein expression as detailed above. In addition to compound concentration and level of light exposure, the reaction is affected by the conditions under which the sample is dosed with UVA light. For example, the required overall concentration for irradiating a population of microbes in a buffered media is going to vary from a population that is cultured in a growth media (e.g. BHI, Tryptase Soy Broth). The photoreaction may be affected by the contents of the growth media, which may interact with the psoralen, thereby requiring a higher overall concentration of the psoralen. In addition, the effective dosing of the microbes may depend on the growth phase of the organism and the presence or absence of compound during the growth phase. In one embodiment, the population of microbes comprises growth media during the psoralen UVA treatment. In one embodiment, the psoralen is added to the population of microbes, the population is cultured to grow the microbes in the presence of psoralen and growth media, and the UVA treatment is performed at some point in the growth phase of the microbes. In one embodiment, the population is grown to an OD of 0.5-1 ( $1 \times 10^7$  to  $1 \times 10^9$  CFU/mL) in the presence of the psoralen prior to irradiation with an appropriate dose of UVA light. Psoralen compounds are effective at concentrations of about 10 pM to 10 mM, also about

100 pM to 1 mM, also about 1 nM to 10  $\mu$ M, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, with the UVA light dose ranging from about 0.1 - 100 J/cm<sup>2</sup>, also about 0.1-20 J/cm<sup>2</sup>, or about 0.5-10 J/cm<sup>2</sup>, 0.5-6 J/cm<sup>2</sup> or about 2-6 J/cm<sup>2</sup>. In one embodiment, the microbe is treated in the presence of growth media at psoralen concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. In one embodiment, the microbe treated in the presence of growth media is grown to an OD of 0.5-1 in the presence of psoralen at concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. Following the growth to an OD of 0.5-1, the microbe population is irradiated with UVA light at a dose ranging from about 0.1 - 100 J/cm<sup>2</sup>, also about 0.1-20 J/cm<sup>2</sup>, or about 0.5-10 J/cm<sup>2</sup>, 0.5-6 J/cm<sup>2</sup> or about 2-6 J/cm<sup>2</sup>.

#### Microbes containing heterologous nucleic acid sequences

[0100] Microbes can be altered to include a heterologous nucleic acid sequence that can be expressed by the microbe. The heterologous sequence can encode at least one specific protein antigen. The microbes may be altered by methods known to one skilled in the art [Sambrook and Russell, Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, (2000)]. The microbes can be altered to contain one or more sequences that encode one or more antigens. The heterologous nucleic acid sequence encoding a specific antigen is not limited to an exact nucleic acid sequence but is of a sequence that is sufficient to provide the expression of an antigen that will elicit the desired immune response when administered to an individual. The heterologous sequence can be expressed as an antigen related to a particular disease. The microbe expressing such antigens can be used as a vaccine, wherein the vaccine may be used as a preventative treatment or a therapeutic treatment. Diseases that can be treated by such vaccines include infectious diseases, autoimmune diseases, allergies, cancers and other hyperproliferative diseases.

[0101] The microbes of the invention may be altered to contain a heterologous nucleic acid sequence encoding a specific tumor antigen. A large number of tumor specific antigens that are recognized by T cells have been identified [Renkvist et al., Cancer Immunol Immunother 50:3-15 (2001)]. These tumor antigens may be differentiation antigens (e.g., PSMA, Tyrosinase, gp100), tissue-specific antigens (e.g. PAP, PSA), developmental antigens, tumor-associated viral antigens (e.g. HPV 16 E7), cancer-testis antigens (e.g. MAGE, BAGE, NY-ESO-1), embryonic antigens (e.g. CEA, alpha-

fetoprotein), oncoprotein antigens (e.g. Ras, p53), over-expressed protein antigens (e.g. ErbB2 (Her2/Neu), MUC1), or mutated protein antigens. The tumor antigens that may be encoded by the heterologous nucleic acid sequence include, but are not limited to, 707-AP, Annexin II, AFP, ART-4, BAGE,  $\beta$ -catenin/m, BCL-2, bcr-abl, bcr-abl p190, bcr-abl p210, BRCA-1, BRCA-2, CAMEL, CAP-1, CASP-8, CDC27/m, CDK-4/m, CEA, CT9, CT10, Cyp-B, Dek-cain, DAM-6 (MAGE-B2), DAM-10 (MAGE-B1), ELF2M, EphA2, ETV6-AML1, G250, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7B, GAGE-8, GnT-V, gp100, HAGE, HER2/neu, HLA-A\*0201-R170I, HPV-E7, HSP70-2M, HST-2, hTERT, hTERT, iCE, inhibitors of apoptosis (e.g. survivin), KIAA0205, LAGE, LAGE-1, LDLR/FUT, MAGE-1, MAGE-2, MAGE-3, MAGE-6, MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, MAGE-B5, MAGE-B6, MAGE-C2, MAGE-C3, MAGE-D, MART-1, MART-1/Melan-A, MC1R, MDM-2, mesothelin, Myosin/m, MUC1, MUC2, MUM-1, MUM-2, MUM-3, neo-polyA<sup>-</sup> polymerase, NA88-A, NY-ESO-1, NY-ESO-1a (CAG-3), PAGE-4, PAP, Proteinase 3 (PR3), P15, p190, Pml/RAR $\alpha$ , PRAME, PSA, PSM, PSMA, RAGE, RAS, RCAS 1, RU1, RU2, SAGE, SART-1, SART-2, SART-3, SP17, SPAS-1, TEL/AML1, TPI/m, Tyrosinase, TARP, TRP-1 (gp75), TRP-2, TRP-2/INT2, WT-1, and alternatively translated NY-ESO-ORF2 and CAMEL proteins, derived from the NY-ESO-1 and LAGE-1 genes. The microbes of the present invention encompass any tumor antigen that can elicit a tumor-specific immune response, including antigens yet to be identified. The microbes may be altered to contain more than one heterologous sequence encoding more than one tumor antigen. Preferred antigens include mesothelin [Argani et al., Clin Cancer Res. 7(12):3862-8 (2001)], Sp17 [Lim et al., Blood. 97(5):1508-10 (2001)], gp100 [Kawakami et al., Proc. Natl. Acad. Sci. USA 91:6458 (1994)], PAGE-4 [Brinkmann et al., Cancer Res. 59(7):1445-8 (1999)], TARP [Wolfgang et al., Proc. Natl. Acad. Sci. USA 97(17):9437-42 (2000)], EphA2 [Tatsumi et al., Cancer Res. 63(15):4481-9 (2003)], PR3 [Muller-Berat et al., Clin. Immunol. Immunopath. 70(1):51-9 (1994)] and SPAS-1 [U.S. Patent Application Publication Number 20020150588].

[0102] In one embodiment of the invention, the heterologous antigen expressed by the modified microbe is CEA. CEA is a 180-kDa membrane intercellular adhesion glycoprotein that is over-expressed in a significant proportion of human tumors, including 90% of colorectal, gastric, and pancreatic, 70% of non-small cell lung cancer, and 50% of breast cancer (Hammarstrom, *Semin. Cancer Biol.*, 9:67-81). A variety of

immunotherapeutics such as anti-idiotypic monoclonal antibody mimicking CEA (Foon et al., *Clin. Cancer Res.*, 87:982-90 (1995), or vaccination using a recombinant vaccinia virus expressing CEA (Tsang et al., *J. Natl. Cancer Inst.*, 87:982-90 (1995)) have been investigated, unfortunately, however, with limited success. Nonetheless, investigators have identified a HLA\*0201-restricted epitope, CAP-1(CEA605-613), that is recognized by human T cell lines that were generated from vaccinated patients. Vaccination of patients with DC pulsed with this epitope failed to induce clinical responses (Morse et al., *Clin. Cancer Res.*, 5:1331-8 (1999)). Recently, a CEA605-613 peptide agonist was identified with a heteroclitic aspartate to asparagine substitution at position 610 (CAP1-6D). Although this amino acid substitution did not alter MHC binding affinity of this peptide, the use of the altered peptide ligand (APL) resulted in improved generation of CEA-specific cytotoxic T lymphocytes (CTL) *in vitro*. CAP1-6D-specific CTL maintained their ability to recognize and lyse tumor cells expressing native CEA (Zaremba et al., *Cancer Res.*, 57: 4570-7 (1997); Salazar et al., *Int. J. Cancer*, 85:829-38 (2000)). Fong et al. demonstrated induction of CEA-specific immunity in patients with colon cancer vaccinated with Flt3-ligand expanded DC incubated with this APL. Encouragingly, 2 of 12 patients after vaccination experienced dramatic tumor regressions that correlated with the induction of peptide-MHC tetramer<sup>+</sup> T cells (Fong et al., *Proc. Natl. Acad. Sci. U.S.A.*, 98:8809-14 (2001)). Taken together, this work provides significant validation for CEA-targeted immunotherapy for colorectal cancer.

[0103] In another embodiment, the heterologous antigen expressed by the modified microbe is proteinase-3 or is derived from proteinase-3. For instance, in one embodiment, the antigen comprises the HLA-A2.1-restricted peptide PR1 (aa 169-177; VLQELNVTV (SEQ ID NO:50)). Information on proteinase-3 and/or the PR1 epitope is publicly available in the following references: US Patent No. 5,180,819, Molldrem, et al., *Blood*, 90:2529-2534 (1997); Molldrem et al., *Cancer Research*, 59:2675-2681 (1999); Molldrem, et al., *Nature Medicine*, 6:1018-1023 (2000); and Molldrem et al., *Oncogene*, 21: 8668-8673 (2002).

[0104] Accordingly, in some embodiments, the modified microbe comprises a nucleic acid molecule encoding an antigen such as mesothelin, SPAS-1, proteinase-3, EphA2, SP-17, gp100, PAGE-4, TARP, Her-2/neu, WT-1, NY-ESO-1, PSMA, K-ras, or CEA, or an antigen derived from one of those proteins. In some embodiments, the modified microbe comprises a nucleic acid molecule encoding an antigen such as mesothelin, SPAS-1, proteinase-3, SP-17, gp100, PAGE-4, TARP, WT-1, NY-ESO-1 or

CEA, or an antigen derived from one of those proteins. In some embodiments, the modified microbe comprises a nucleic acid molecule encoding human mesothelin, or an antigen derived from human mesothelin. In other embodiments, the modified microbe comprises a nucleic acid molecule encoding human EphA2, or derived from human EphA2.

[0105] The microbes of the invention may be altered to contain a heterologous nucleic acid sequence encoding a specific infectious disease antigen. In one embodiment, the antigen is derived from a human or animal pathogen. The pathogen is optionally a virus, bacterium, fungus, or a protozoan. For instance, the antigen may be a viral or fungal or bacterial antigen.

[0106] For instance, the antigen may be derived from Human Immunodeficiency virus (such as gp 120, gp 160, gp41, gag antigens such as p24gag and p55gag, as well as proteins derived from the pol, env, tat, vif, rev, nef, vpr, vpu and LTR regions of HIV), Feline Immunodeficiency virus, or human or animal herpes viruses. In one embodiment, the antigen is derived from herpes simplex virus (HSV) types 1 and 2 (such as gD, gB, gH, Immediate Early protein such as ICP27), from cytomegalovirus (such as gB and gH), from Human Metapneumovirus, from Epstein-Barr virus or from Varicella Zoster Virus (such as gPI, II or III). (See, e. g. Chee et al. (1990) Cytomegaloviruses (J. K. McDougall, ed., Springer Verlag, pp. 125-169; McGeoch et al. (1988) J. Gen. Virol. 69: 1531-1574; U.S. Pat. No. 5,171,568; Baer et al. (1984) Nature 310: 207-211; and Davison et al. (1986) J. Gen. Virol. 67: 1759-1816.)

[0107] In another embodiment, the antigen is derived from a hepatitis virus such as hepatitis B virus (for example, Hepatitis B Surface antigen), Hepatitis A virus, hepatitis C virus, delta hepatitis virus, hepatitis E virus, or hepatitis G virus. See, e. g., WO 89/04669; WO 90/11089; and WO 90/14436. The HCV genome encodes several viral proteins, including E1 and E2. See, e. g., Houghton et al., *Hepatology* 14: 381-388(1991).

[0108] An antigen that is a viral antigen is optionally derived from a virus from any one of the families Picornaviridae (e. g., polioviruses, rhinoviruses, etc.); Caliciviridae; Togaviridae (e. g., rubella virus, dengue virus, etc.); Flaviviridae; Coronaviridae; Reoviridae (e. g., rotavirus, etc.); Birnaviridae; Rhabdoviridae (e. g., rabies virus, etc.); Orthomyxoviridae (e. g., influenza virus types A, B and C, etc.); Filoviridae; Paramyxoviridae (e. g., mumps virus, measles virus, respiratory syncytial virus, parainfluenza virus, etc.); Bunyaviridae; Arenaviridae; Retroviridae (e. g., HTLV-I; HTLV-11; HIV-1 (also known as HTLV-111, LAV, ARV, hTLR, etc.)), including but not limited to antigens from the isolates HIV11b, HIVSF2, HTVLAV, HIVLAI, HIVMN);



HIV-1CM235, HIV-1; HIV-2, among others; simian immunodeficiency virus (SIV); Papillomavirus, the tick-borne encephalitis viruses; and the like. See, e. g. Virology, 3rd Edition (W. K. Joklik ed. 1988); Fundamental Virology, 2.sup.nd Edition (B. N. Fields and D. M. Knipe, eds. 1991), for a description of these and other viruses.

[0109] In some alternative embodiments, the antigen is derived from bacterial pathogens such as *Mycobacterium*, *Bacillus*, *Yersinia*, *Salmonella*, *Neisseria*, *Borrelia* (for example, OspA or OspB or derivatives thereof), *Chlamydia*, or *Bordetella* (for example, P.69, PT and FHA), or derived from parasites such as plasmodium or Toxoplasma. In one embodiment, the antigen is derived from *Mycobacterium tuberculosis* (e.g. ESAT-6, 85A, 85B, 72F), *Bacillus anthracis* (e.g. PA), or *Yersinia pestis* (e.g. F1, V). In addition, antigens suitable for use in the present invention can be obtained or derived from known causative agents responsible for diseases including, but not limited to, Diphtheria, Pertussis, Tetanus, Tuberculosis, Bacterial or Fungal Pneumonia, Otitis Media, Gonorrhea, Cholera, Typhoid, Meningitis, Mononucleosis, Plague, Shigellosis or Salmonellosis, Legionaire's Disease, Lyme Disease, Leprosy, Malaria, Hookworm, Onchocerciasis, Schistosomiasis, Trypanosomiasis, Leishmaniasis, Giardia, Amoebiasis, Filariasis, Borelia, and Trichinosis.

[0110] The microbes of the invention may be altered to contain a heterologous nucleic acid sequence encoding an autoimmune disease-specific antigen. In a T cell mediated autoimmune disease, a T cell response to self antigens results in the autoimmune disease. The type of antigen for use in treating an autoimmune disease with the vaccines of the present invention might target the specific T cells responsible for the autoimmune response. For example, the antigen may be part of a T cell receptor, the idiotype, specific to those T cells causing an autoimmune response, wherein the antigen incorporated into a vaccine of the invention would elicit an immune response specific to those T cells causing the autoimmune response. Eliminating those T cells would be the therapeutic mechanism to alleviating the autoimmune disease. Another possibility would be to incorporate an antigen that will result in an immune response targeting the antibodies that are generated to self antigens in an autoimmune disease or targeting the specific B cell clones that secrete the antibodies. For example, an idiotype antigen may be incorporated into the microbe that will result in an anti-idiotypic immune response to such B cells and/or the antibodies reacting with self antigens in an autoimmune disease. Autoimmune diseases that may be treatable with vaccine microbes of the present invention include, but are not limited to, rheumatoid arthritis, multiple sclerosis, Crohn's disease, lupus, myasthenia gravis, vitiligo, scleroderma, psoriasis, pemphigus vulgaris, fibromyalgia, colitis and diabetes. A similar

approach may be taken for treating allergic responses, where the antigens incorporated into the vaccine microbe target either T cells, B cells or antibodies that are effective in modulating the allergic reaction. In some autoimmune diseases, such as psoriasis, the disease results in hyperproliferative cell growth with expression of antigens that may be targeted as well. Such an antigen that will result in an immune response to the hyperproliferative cells is considered.

[0111] Rather than targeting the malfunctioning cells of a disease, the microbes of the present invention comprise antigens that target unique disease associated protein structures. One example of this is the targeting of antibodies, B cells or T cells using idiotype antigens as discussed above. Another possibility is to target unique protein structures resulting from a particular disease. An example of this would be to incorporate an antigen that will generate an immune response to proteins that cause the amyloid plaques observed in diseases such as Alzheimer's disease, Creutzfeldt-Jakob disease (CJD) and Bovine Spongiform Encephalopathy (BSE). While this approach may only provide for a reduction in plaque formation, it may be possible to provide a curative vaccine in the case of diseases like CJD. This disease is caused by an infectious form of a prion protein. The vaccine incorporates an antigen to the infectious form of the prion protein such that the immune response generated by the vaccine may eliminate, reduce, or control the infectious proteins that cause CJD.

#### Microbes containing mutations

[0112] In one embodiment, the invention includes a vaccine comprising a microbe wherein the nucleic acid of the microbe is modified so that the proliferation of the microbe is attenuated, wherein the microbial population is still able to express a desired antigen to an extent that is sufficient to elicit an immune response, and wherein the microbe is further attenuated by at least one genetic mutation. The mutation in the microbe may affect a variety of features of the microbe. In some cases, the mutation affects the ability of the microbe to invade certain cells. For example, certain intracellular bacteria can invade a variety of cell types depending on receptors present on the bacteria. The mutation may alter the expression of certain receptors so that the bacteria is taken up by some cell types but not others. As an example of this, *Listeria* is typically taken up by phagocytic cells and also actively invades non-phagocytic cells (e.g. hepatic cells). A mutation of *Listeria* may be used in which the invasion of non-phagocytic cells is significantly reduced or eliminated while the uptake by phagocytic cells is sufficiently active. Such a mutation may provide for a better immune response as the vaccine would be preferentially taken up by phagocytic

cells, which are important in presenting the bacterial antigens to the immune system. It is understood that the mutation can be to any gene that results in an attenuation of the ability of the microbe to invade certain cell types, and that this is exemplified by mutations to internalin genes in *Listeria* (e.g. *inlA*, *inlB*). Similar genes may exist (e.g. invasin genes in *Salmonella*, *Bacillus anthracis*, and *Yersinia*) in other bacteria, and mutations in these genes are encompassed by the present invention. The mutation might impact other features of the microbe, such as a virulence factor or a gene that allows for growth and spreading, thereby reducing the virulence of the microbe. For example, a mutation in the *actA* gene of *Listeria* causes a deficiency in the polymerization of host cell actin, which inhibits the ability of the *Listeria* to spread to other cells. A mutation in the *hly* gene of *Listeria* (listeriolysin (LLO) protein) impacts the ability of the *Listeria* to escape the phagolysosome of an infected cell. A mutation in either the *plcA* or *plcB* genes of *Listeria* impacts the ability of the *Listeria* to spread from cell to cell. A mutation in the *yop* gene of *Yersinia* affects the ability of the *Yersinia* to prevent phagocytosis by macrophages. In another embodiment the genetic mutation attenuates the expression of certain antigens, for example, antigens that would normally result in an immune response to the microbe itself. Such a mutation may be useful if the microbe is used as a vaccine comprising a heterologous antigen in order to stimulate a strong immune response to the heterologous antigen but with a reduced immune response to the delivery microbe compared to the non-mutated microbe. In one embodiment, the microbe is attenuated by a mutation in more than one gene. In one embodiment, one of the mutations is in an internalin gene of *Listeria* or a similar gene in other bacteria. In one embodiment, the mutation is in one or more of an internalin gene of *Listeria* or similar gene in other bacteria. In one embodiment, one of the mutations is in the *actA* gene. In one embodiment, the microbe comprises *Listeria monocytogenes* with mutations in the *actA* gene and one or more internalin genes. In a preferred embodiment, the *Listeria monocytogenes* comprises a mutation in the *actA* gene and the *inlB* gene, preferably the *Listeria monocytogenes* comprises an *actA/inlB* deletion mutant (which is alternatively referred to herein as either  $\Delta actA \Delta inlB$  or *actA<sup>-</sup>inlB<sup>-</sup>*). The sequences of a variety of *Listeria* genes including those described herein are found in Genbank accession no. NC\_003210.

[0113] The microbe might contain a mutation that significantly reduces the ability of the microbe to repair modifications to their nucleic acid. Such a mutation could be in any of a variety of genes that are involved in the DNA repair mechanisms of microbes

[Aravind et al., Nucleic Acids Research 27(5):1223–1242 (1999)]. Microbes that are deficient in their ability to repair damage to their nucleic acid provide an added level of safety and efficacy to the use of the microbes of the present invention. Using the appropriate repair deficient mutants, the microbes are exquisitely sensitive to nucleic acid modification. The nucleic acid of the microbes may be modified to a lesser degree yet still ensure the desired amount of attenuation of proliferation. This provides a larger window of efficacy in which to operate so that the expression of the microbial nucleic acid is sufficient to generate the desired proteins. In the case where *de novo* antigen expression is required, this provides a vaccine that will elicit an effective immune response. It also provides an added level of safety as the level of attenuation of proliferation achieved can not be compromised by repair of the modified nucleic acid. In another embodiment, the genetic mutation alters the susceptibility of the microbe to treatment with a nucleic acid targeted compound, for example by altering the permeability of the microbe to the compound or by altering the ability of the compound to access and bind the microbial nucleic acid. Such mutations may also impact the efficacy of the process of attenuating proliferation while leaving microbial gene expression substantially unaffected.

[0114] To illustrate the advantages of using a repair deficient mutant, one can consider the mechanism of the attenuation of microbial proliferation. The microbial nucleic acid is modified either by strand breakage or pyrimidine dimers, or by chemical modifications such as monoadducts or crosslinks. If the mechanisms for repair of these modifications are intact, a certain number of modifications will be required in order to achieve sufficient attenuation of proliferation. The greater the modification of nucleic acid, the greater the reduction in protein expression. Even though the levels of modification required to attenuate proliferation are much lower than the levels required to stop protein expression, protein expression will still be reduced to some extent, possibly to an unacceptable level. The use of repair deficient mutants significantly reduces the levels needed to attenuate proliferation such that a lower modification level will result in adequate attenuation of proliferation. Since the nucleic acid modification is much lower, the expression of proteins will be less affected, providing for a higher level of expression of the protein of interest. Such repair deficient mutants may be particularly useful in the preparation of vaccines, such as vaccines to the microbe itself, where the safety of the vaccine can be increased by a slight modification of the nucleic acid, leaving a sufficiently high level of protein expression, in particular the antigen to which the immune response is targeted. In one embodiment the repair deficient mutant lacks the ability to make PhrB (a

photolyase), which repairs pyrimidine dimers. For example, the mutation may be in the *phrB* gene, or a functionally equivalent gene, depending on the genus and species of the microbe. Such a mutant could be used in conjunction with ultraviolet irradiation (e.g. UVB, UVC) of the microbe to produce pyrimidine dimers in the microbial nucleic acid. In one embodiment the repair deficient mutant is unable to repair interstrand crosslinks. Such mutants include, but are not limited to, mutations in *uvr* genes, i.e. *uvrA*, *uvrB*, *uvrC*, and *uvrD* genes as well as *recA* genes, or functionally equivalent genes, depending on the genus and species of the microbe. The mutations may be in one or more of these genes. These mutations result in attenuation in the activity of the corresponding enzymes UvrA (an ATPase), UvrB (a helicase), UvrC (a nuclease), UvrD (a helicase II) and RecA (a recombinase). These mutants would be used in conjunction with a crosslinking compound, such as a psoralen. Since the microbial nucleic acid is crosslinked in some locations, and these crosslinks can not be repaired, the microbe is unable to replicate as the original strands of nucleic acid can not be separated. Since they can not be repaired, very few crosslinks are needed, the microbial nucleic acid is for the most part accessible for transcription, and protein expression is not altered significantly. In a preferred embodiment, a population of repair deficient microbial mutants that are unable to repair interstrand crosslinks are suitably crosslinked such that essentially every microbe in the population contains at least one crosslink, such that attenuation of replication is essentially complete, wherein the microbial gene expression of the population is sufficiently active. In one embodiment, a mutation in the *recA* gene is a conditional mutation. In such a mutation, the mutation in the *recA* gene results in the attenuation in the activity of *recA* only under certain conditions (i.e. non-permissive conditions), such as a suitable pH or temperature of the microbial population. A microbe comprising a conditional *recA* mutation can be cultured under permissive conditions in order to grow sufficient levels of the microbe and then placed under non-permissive conditions for treatment to modify the nucleic acid, then stored under non-permissive conditions such that the nucleic acid damage is not adequately repaired. As an example of this, a *recA* temperature sensitive mutant is grown at 30 °C, where it grows well, and is treated to modify the nucleic acid at 42 °C, which is non-permissive for *recA* such that it is very sensitive to treatment, such as psoralen crosslinking. While the treated microbe may be stored under non-permissive conditions, it is possible that upon vaccination, the conditions may permit expression of *recA*, resulting in some repair and presenting a safety issue. It is possible to construct the microbe such that the *recA* is under the control of the lac repressor, such that growth of the

strain can be induced by isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) when growth is desirable, prior to the inactivation and/or immunization steps. The possibility of *recA* expression can then be eliminated for the inactivation and/or immunization steps by withholding further IPTG from the strain and/or eliminating IPTG from the strain's environment.

[0115] In one embodiment, the microbe comprises at least one mutation that significantly reduces the ability of the microbe to repair modifications to their nucleic acid in combination with at least one mutation not related to repair mechanisms. The mutation that is not related to repair mechanisms may affect a variety of features of the microbe, such as the ability of the microbe to invade certain cells, a mutation in a virulence factor or a gene that allows for growth and spreading, or a mutation that attenuates the expression of certain antigens. Such mutations are discussed above and include, but are not limited to, mutations in internalin genes (e.g. *inlB*), *actA* gene, *hly* gene, *plcA* gene, or *plcB* gene of *Listeria*, invasion genes (e.g. *Salmonella*, *Bacillus anthracis*, and *Yersinia*) or the *yop* gene of *Yersinia*. In one embodiment, the microbe comprises *Listeria monocytogenes* having a mutation in the *actA* gene. In one embodiment, the *Listeria monocytogenes* comprises a mutation in the *actA* gene and in an internalin gene. In one embodiment, the *Listeria monocytogenes* comprises an *actA* mutation and a *uvrAB* mutation, preferably *actA/uvrAB* deletion mutations (which may be referred to as either  $\Delta actA \Delta uvrAB$  or *actA<sup>-</sup>uvrAB<sup>-</sup>*). In one embodiment, the *Listeria monocytogenes* comprises an *actA* mutation, an *inlB* mutation, and a *uvrAB* mutation, preferably *actA/inlB/uvrAB* deletion mutations. In some other embodiments, the microbe comprises *Bacillus anthracis* having a *uvrAB* mutation, such as a deletion.

[0116] In another embodiment, the invention provides an isolated mutant *Listeria* strain, such as a mutant *Listeria monocytogenes* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the mutant *Listeria* strain is defective with respect to at least one DNA repair enzyme (such as UvrA and/or UvrB). In some embodiments, the mutant *Listeria* strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the mutant strain is the *Listeria monocytogenes*  $\Delta actA \Delta uvrAB$  strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563. In other embodiments, strain is a mutant of the *Listeria monocytogenes*  $\Delta actA \Delta uvrAB$  strain deposited with the American

Type Culture Collection (ATCC) and identified by accession number PTA-5563, wherein the mutant of the deposited strain is defective with respect to UvrA, UvrB, and ActA.

[0117] In some embodiments, the invention provides a free-living microbe which is defective with respect to at least one DNA repair enzyme (relative to wild type). In some embodiments, the microbe that is defective with respect to at least one DNA repair enzyme is attenuated for DNA repair relative to wild type. In some embodiments, the capacity of the microbe for DNA repair is reduced by at least about 10%, at least about 25%, at least about 50%, at least about 75%, or at least about 90% relative to wild type. Methods for assessing the ability of a microbe to effect DNA repair are well known to those of ordinary skill in the art. In some embodiments, the microbe is defective with respect to one or more of the following enzymes: PhrB, UvrA, UvrB, UvrC, UvrD, and RecA. In some embodiments, the microbe is defective with respect to UvrA, UvrB, or both enzymes. In some embodiments, the microbe is defective with respect to RecA, or a functional equivalent of Rec A. In some embodiments, the microbe comprise a genetic mutation in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*. In some embodiments, the microbe comprises genetic mutations in both *uvrA* and *uvrB*, or in functional equivalents of both *uvrA* and *uvrB*. In some embodiments, the microbe comprises a genetic mutation in *recA*. In some embodiments, the microbe is a bacterium. For instance, in some embodiments, the microbe is *Mycobacterium tuberculosis*, *Listeria monocytogenes*, or *Bacillus anthracis*.

[0118] The invention also provides an isolated mutant *Listeria monocytogenes* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the mutant strain is defective with respect to at least one DNA repair enzyme (such as UvrA and/or UvrB). In some embodiments, the mutant strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the *uvrA* gene, the *uvrB* gene, or both genes are deleted. In some embodiments, the mutant strain is attenuated with respect to RecA. In some embodiments, the mutant strain comprises a genetic mutation in the *recA* gene. In some embodiments, the mutant microbe is the *Listeria monocytogenes actA/uvrAB* strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563, or a mutant of the deposited strain which is defective with respect to UvrA, UvrB, and ActA.

[0119] The invention also provides an isolated mutant *Bacillus anthracis* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid. In some

embodiments, the mutant strain is defective with respect to at least one DNA repair enzyme (such as UvrA and/or UvrB). In some embodiments, the mutant strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the *uvrA* gene (SEQ ID NO:18), the *uvrB* gene (SEQ ID NO:19), or both genes are deleted. In some embodiments, the mutant strain is attenuated with respect to RecA. In some embodiments, the mutant strain comprises a genetic mutation in the *recA* gene. In some embodiments, the mutant strain comprises a mutation in the *recA* gene that makes expression of the *recA* protein temperature sensitive. In some alternative embodiments, a mutant strain of *B. anthracis* is constructed which is under control of the lac repressor (inducible by IPTG), permitting expression of *recA* during growth, but not during inactivation (such as with S-59/UVA) and/or post-immunization. In some embodiments, the mutant strain comprises one or more mutations in the *lef* gene, *cya* gene, or both genes, that decreases the toxicity of the strain.

[0120] As with any microbe of the invention, the modification of the DNA of the repair deficient (e.g. uvr deficient) bacteria with psoralen can be controlled by adjusting the compound concentration, reaction conditions and light dose. The appropriate concentration, reaction conditions and light dose are determined by assessing their effects on replication and protein expression as detailed above. The use of repair deficient mutants provides an additional level of control of proliferation while maintaining adequate protein expression such that the parameters of concentration, reaction conditions and light dose can be adjusted over a wider range of conditions to provide a suitable population of microbes. For example, there will be a broader range of nucleic acid modification density over which proliferation can be completely inhibited without significantly affecting protein expression. The minimum level of modification required to completely inhibit repair deficient strains is much less than for non-repair deficient strains (see Examples 3, 7, 11, and 21). As a result, the modification level can be higher than the minimum level required to stop proliferation (ensuring complete inactivation) yet still be below a level that is detrimental to protein expression. Thus, while the invention is effective for non-repair deficient strains, uvr deficient strains provide greater flexibility in preparing a desirable population of microbes that would be effective as a vaccine. Psoralen compounds are effective at concentrations of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1 nM to 10  $\mu$ M, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, with the UVA light dose ranging from about 0.1 - 100 J/cm<sup>2</sup>, also about 0.1-20 J/cm<sup>2</sup>, also about 0.5-10 J/cm<sup>2</sup>, or about 0.5-6



J/cm<sup>2</sup> or about 2-6 J/cm<sup>2</sup>. In one embodiment, the microbe is treated in the presence of growth media at psoralen concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. In one embodiment, the microbe treated in the presence of growth media is grown to an OD of 0.5-1 in the presence of psoralen at concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. Following the growth to an OD of 0.5-1, the microbe population is irradiated with UVA light at a dose ranging from about 0.1 - 100 J/cm<sup>2</sup>, also about 0.1-20 J/cm<sup>2</sup>, or about 0.5-10 J/cm<sup>2</sup>, 0.5-6 J/cm<sup>2</sup> or about 2-6 J/cm<sup>2</sup>.

[0121] In order to generate primarily psoralen crosslinks in any microbe, particularly uvr deficient mutant bacteria, it is possible to dose the psoralen and UVA light initially to form adducts and follow this with a second dose of UVA light alone to convert some or most of the monoadducts to crosslinks. The psoralen photochemistry is such that absorption of a photon of appropriate energy will first form a monoadduct. Absorption of an additional photon will convert this monoadduct to a crosslink when a furan side monoadduct is appropriately situated in the DNA double helix [Tessman et al., Biochemistry 24:1669-1676 (1985)]. The sample can be dosed with a lower UVA dose at a desired concentration of psoralen and the unreacted psoralen can be removed, e.g. by washing, dialysis or ultrafiltration of the bacteria. The bacteria containing psoralen adducts (monoadducts and crosslinks) can be further dosed with UVA light to convert some or most of the monoadducts to crosslinks without resulting in significant additional adducts to the bacteria. This allows for the controlled addition of a low number of psoralen adducts with the initial light dose, then converting a substantial number of any monoadducts to crosslink with the second dose. This provides for modification of the microbial genome at sufficiently low levels wherein a majority of the adducts formed will be crosslinks. This is particularly effective for blocking replication with uvr deficient mutants. In such embodiments, psoralen compounds are effective at concentrations of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, with the UVA light dose ranging from about 0.1 - 10 J/cm<sup>2</sup>, also about 0.1-2 J/cm<sup>2</sup>, or about 0.5-2 J/cm<sup>2</sup>. Following removal of most of the unreacted psoralen by washing, dialysis or ultrafiltration of the bacteria, the bacteria may be dosed with UVA light ranging from 0.1 - 100 J/cm<sup>2</sup>, also about 0.1-20 J/cm<sup>2</sup>, or about 0.5-10 J/cm<sup>2</sup> or about 2-6 J/cm<sup>2</sup>.

#### Vaccine compositions and *in vivo* efficacy

[0122] Vaccine compositions of the invention comprise a microbe in which the microbial nucleic acid is modified and/or comprise an antigen-presenting cell which has been antigen-loaded and/or activated/matured by infection with a microbe in which the microbial nucleic acid is modified so that the proliferation of the microbe is attenuated, wherein the microbial gene expression is substantially unaffected, as discussed above. The vaccine compositions of the present invention can be used to stimulate an immune response in an individual. The formulations can be administered to an individual by a variety of administration routes. Methods of administration of such a vaccine composition are known in the art, and include oral, nasal, intravenous, intradermal, intraperitoneal, intramuscular, intralymphatic and subcutaneous routes of administration. The vaccine compositions may further comprise additional components known in the art to improve the immune response to a vaccine, such as adjuvants or T cell co-stimulatory molecules. The invention also includes medicaments comprising the pharmaceutical compositions of the invention. An individual to be treated with such vaccines, is any vertebrate, preferably a mammal, including domestic animals, sport animals, and primates, including humans. The vaccine may be administered as a prophylactic, where the individual is vaccinated in order to immunize the individual against a particular disease. While the vaccine can be given to any individual, in some instances, such as with cancer vaccines, the individual treated might be limited to those individuals at higher risk of developing a cancer. The vaccine may also be administered as a therapeutic, where the individual having a particular disease is vaccinated in order to improve the immune response to the disease or a disease related protein. In this embodiment, the vaccine may result in a lessening of the physical symptoms associated with the disease. For example, with cancer vaccines, the vaccination may result in stopping the growth of a tumor, preferably a lessening of the mean tumor volume, more preferably elimination of any tumors. In one embodiment, the mean tumor volume decreases by at least about 5%, also about 10%, also about 25%, also about 50%, also about 75%, also about 90% or about 100%. Similarly, the vaccination may result in stopping the metastases of a tumor, preferably resulting in a reduction in the number of tumor metastases. An additional effect of a cancer vaccine would be an extension of the median survival of the individual. In humans, the median survival may be extended by at least about 3 months, also at least about 6 months, or at least about 12 months.

[0123] Vaccine formulations are known in the art and include numerous additives, such as preservatives, stabilizers, adjuvants, antibiotics, and other substances. Preservatives, such as thimerosal or 2-phenoxy ethanol, are added to slow or stop the

growth of bacteria or fungi resulting from inadvertent contamination, especially as might occur with vaccine vials intended for multiple uses or doses. Stabilizers, such as lactose or monosodium glutamate (MSG), are added to stabilize the vaccine formulation against a variety of conditions, such as temperature variations or a freeze-drying process. Adjuvants, such as aluminum hydroxide or aluminum phosphate, are added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response. Additional materials, such as cytokines, chemokines, and bacterial nucleic acid sequences, like CpG, are also potential vaccine adjuvants. Antibiotics, such as neomycin and streptomycin, are added to prevent the potentially harmful growth of germs. Vaccines may also include a suspending fluid such as sterile water or saline. Vaccines may also contain small amounts of residual materials from the manufacturing process, such as cell or bacterial proteins, egg proteins (from vaccines that are produced in eggs), DNA or RNA, or formaldehyde from a toxoiding process.

[0124] The efficacy of the vaccines can be evaluated in an individual, for example in mice. A mouse model is recognized as a model for efficacy in humans and is useful in assessing and defining the vaccines of the present invention. The mouse model is used to demonstrate the potential for the effectiveness of the vaccines in any individual. Vaccines can be evaluated for their ability to provide either a prophylactic or therapeutic effect against a particular disease. For example, in the case of infectious diseases, a population of mice can be vaccinated with a desired amount of the appropriate vaccine of the invention, where the microbe expresses an infectious disease associated antigen. This antigen can be from the delivery microbe itself or can be a heterologous antigen. The mice can be subsequently infected with the infectious agent related to the vaccine antigen and assessed for protection against infection. The progression of the infectious disease can be observed relative to a control population (either non vaccinated or vaccinated with vehicle only or a microbe that does not contain the appropriate antigen).

[0125] In the case of cancer vaccines, tumor cell models are available, where a tumor cell line expressing a desired tumor antigen can be injected into a population of mice either before (therapeutic model) or after (prophylactic model) vaccination with a microbe of the invention containing the desired tumor antigen. Vaccination with a microbe containing the tumor antigen can be compared to control populations that are either not vaccinated, vaccinated with vehicle, or with a microbe that expresses an irrelevant antigen. In addition, the relative efficacy of the vaccines of the invention can be compared to a population of microbe in which the microbial nucleic acid has not been modified. The

effectiveness of the vaccine in such models can be evaluated in terms of tumor volume as a function of time after tumor injection or in terms of survival populations as a function of time after tumor injection (e.g. Example 4). In one embodiment, the tumor volume in mice vaccinated with nucleic acid modified microbe is about 5%, about 10%, about 25%, about 50%, about 75%, about 90% or about 100% less than the tumor volume in mice that are either not vaccinated or are vaccinated with vehicle or a microbe that expresses an irrelevant antigen. In another embodiment, this differential in tumor volume is observed at least about 10, about 17, or about 24 days following the implant of the tumors into the mice. In one embodiment, the median survival time in the mice vaccinated with nucleic acid modified microbe is at least about 2, about 5, about 7 or at least about 10 days longer than in mice that are either not vaccinated or are vaccinated with vehicle or a microbe that expresses an irrelevant antigen. In addition to an effective immune response to the vaccines of the present invention, the modified microbes provide an added level of safety such that a higher dose of the microbe may be administered relative to the corresponding unmodified microbe. In one embodiment of the invention, the vaccination with the nucleic acid modified microbe is done at a dose of microbes that is the same as the dose of the corresponding unmodified microbe. In another embodiment, the vaccination of nucleic acid modified microbe is safely dosed at a level that is at least about 2, about 5, about 10, about  $10^2$ , about  $10^3$ , or at least about  $10^4$  fold higher than the vaccination dose of the corresponding unmodified microbe, wherein the resulting tumor volume and median survival times discussed above are observed for the nucleic acid modified microbe.

#### Methods of use

[0126] A variety of methods of using the modified microbes, antigen-presenting cells, vaccines, and pharmaceutical compositions described herein are provided by the present invention. For instance, methods of using the modified microbes, antigen-presenting cells, vaccines, and pharmaceutical compositions described herein to induce immune responses and/or to treat or prevent disease are provided. Method of using the modified microbes and/or mutant strain to prepare vaccines and other compositions are also provided.

[0127] For instance, in one aspect, the invention provides a method of inducing an immune response in a host to an antigen, comprising administering to the host an effective amount of a composition comprising a free-living microbe that expresses the antigen, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for

proliferation. In some embodiments, the composition comprising the microbe is a vaccine. In some embodiments, the composition comprising the microbe is a professional antigen-presenting cell. The antigen may be heterologous or autologous to the microbe as described above. In some embodiments, the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid.

[0128] The invention also provides a method of inducing an immune response in a host to an antigen, comprising administering to the host an effective amount of a composition comprising a mutant strain of *Listeria monocytogenes* that expresses the antigen, wherein the mutant strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid. The antigen may be a Listerial or non-Listerial antigen. In some embodiments, the nucleic acid of the *Listeria* has been modified so that the microbe is attenuated for proliferation (e.g., by S-59/UVA treatment).

[0129] The invention also provides a method of inducing an immune response in a host to an antigen, comprising administering to the host an effective amount of a composition comprising a mutant strain of *Bacillus anthracis* that expresses the antigen, wherein the mutant strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the nucleic acid of the *Bacillus* has been modified so that the microbe is attenuated for proliferation (e.g., by S-59/UVA treatment).

[0130] The invention also provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of a composition comprising a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. In some embodiments, the composition comprising the microbe is a vaccine. In some embodiments, the composition comprising the microbe is a professional antigen-presenting cell.

[0131] The invention also provides a method of preventing or treating a disease in a host, comprising administering to the host an effective amount of a composition comprising a mutant strain of *Listeria monocytogenes*, wherein the mutant strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the nucleic acid of the *Listeria* has been modified so that the microbe is attenuated for proliferation (e.g., by S-59/UVA treatment). In some embodiments, the disease is an infectious disease. In other embodiments, the disease is cancer.

[0132] The invention also provides a method of preventing or treating disease in a host, comprising administering to the host an effective amount of a composition comprising a mutant strain of *Bacillus anthracis*, wherein the mutant strain comprises a genetic

mutation that attenuates its ability to repair its nucleic acid. In some embodiments, the nucleic acid of the *Bacillus* has been modified so that the microbe is attenuated for proliferation (e.g., by S-59/UVA treatment).

[0133] The invention also provides a free-living microbe for medical use, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation and/or the microbe is defective with respect to a DNA repair enzyme. It is understood that medical use encompasses both therapeutic and preventative medical applications (e.g., for use as a vaccine). In some embodiments, the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation. In some embodiments, the microbe is *Listeria monocytogenes* or *Bacillus anthracis*.

[0134] In other aspects, the invention provides a professional antigen-presenting cell for medical use, wherein the antigen-presenting cell comprises a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation and/or the microbe is defective with respect to a DNA repair enzyme. In some embodiments, the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation. In some embodiments, the microbe is *Listeria monocytogenes* or *Bacillus anthracis*.

[0135] The invention also provides a mutant *Listeria monocytogenes* strain for medical use, wherein the mutant *Listeria monocytogenes* strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid.

[0136] In addition, the invention provides a mutant *Bacillus anthracis* strain for medical use, wherein the mutant *Bacillus anthracis* strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid.

[0137] The invention further provides the use of a free-living microbe, wherein the nucleic acid has been modified so that the microbe is attenuated for proliferation, for the manufacture of a medicament for a disease unrelated and/or not caused by the free-living microbe. In some embodiments, the disease is cancer. In some embodiments, the disease is an infectious disease unrelated to the free-living microbe.

[0138] The invention further provides the use of a free-living microbe for the manufacture of a medicament for a disease unrelated and/or not caused the microbe, wherein the microbe is defective with respect to at least one DNA repair enzyme. In some

embodiments, the disease is cancer. In some embodiments, the disease is an infectious disease unrelated to the microbe.

[0139] Additionally, the invention provides the use of a professional antigen-presenting cell, wherein the antigen-presenting cell comprises a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation and/or wherein the microbe is defective with respect to at least one DNA repair enzyme, for the manufacture of a medicament for a disease unrelated and/or not caused by the free-living microbe. In some embodiments, the disease is cancer. In some embodiments, the disease is an infectious disease unrelated to the free-living microbe.

[0140] The invention further provides the use of a mutant strain of *Listeria monocytogenes*, wherein the mutant *Listeria monocytogenes* strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid, for the manufacture of a medicament for a disease unrelated and/or not caused by *Listeria monocytogenes*. In some embodiments, the disease is cancer. In some embodiments, the disease is an infectious disease unrelated to the *Listeria monocytogenes*.

[0141] In another aspect, the invention provides a method of activating naïve T cells, comprising contacting the naïve T cells with a professional antigen-presenting cell under suitable conditions and for a sufficient time to activate the naïve T cells, wherein the antigen-presenting cell comprises a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. The contacting step of this method may be performed either *in vitro* or *in vivo*. Suitable conditions and a sufficient time for activating the naïve T-cells would be known to one of ordinary skill of the art. In addition, examples of such conditions are provided in the specific Examples, below.

[0142] A method of loading professional antigen-presenting cells with an antigen is also provided. The method comprises contacting the professional antigen-presenting cells with a free-living microbe that comprises a nucleic acid sequence encoding the antigen, under suitable conditions and for a sufficient time to load the professional antigen-presenting cells (e.g., dendritic cells), wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation and/or the microbe is defective with respect to at least one DNA repair enzyme. The contacting step of the method may be performed *in vitro*, *ex vivo*, or *in vivo*. The antigen may be heterologous or autologous to the microbe. Suitable conditions and a sufficient time for loading antigen-presenting cells

would generally be known to one of ordinary skill of the art. In addition, examples of such conditions are provided in the specific Examples, below.

[0143] In another aspect, the invention provides a method of activating and/or maturing professional antigen-presenting cells comprising contacting the professional antigen-presenting cells (*in vitro*, *ex vivo*, and/or *in vivo*) with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, under suitable conditions and for a sufficient time to activate and/or bring to maturation the professional antigen-presenting cells, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation. The contacting step of the method may be performed either *in vitro* or *in vivo*. The antigen may be heterologous or autologous to the microbe. Suitable conditions and a sufficient time for activating antigen-presenting cells and/or bringing antigen-presenting cells to maturation would generally be known to one of ordinary skill of the art. In addition, examples of such conditions are provided in the specific Examples, below.

[0144] In another aspect, the invention provides a method of preventing or treating a disease in a host, comprising the following steps. (a) loading professional antigen-presenting cells with an antigen by contacting the cells with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) administering an effective amount of a composition comprising the loaded professional antigen-presenting cells to the host.

[0145] In still another aspect, the invention provides a method of inducing an immune response to an antigen in a host, comprising the following steps. (a) loading professional antigen-presenting cells with the antigen by contacting the cells with a free-living microbe that comprises a nucleic acid sequence encoding the antigen, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) administering an effective amount of a composition comprising the loaded professional antigen-presenting cells to the host.

#### Kits

[0146] The invention further provides kits (or articles of manufacture) comprising the modified microbes and mutant strains of the present invention.

[0147] In one aspect, the invention provides a kit comprising both (a) a composition comprising a mutant *Listeria monocytogenes* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid, a mutant *Bacillus anthracis* strain comprising



a genetic mutation that attenuates its ability to repair its nucleic acid, or a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) instructions for the use of the composition in the prevention or treatment of a disease in a host. In some embodiments, the instructions are on a label. In other embodiments, the instructions are on an insert contained within the kit.

[0148] In another aspect, the invention provides a kit comprising both (a) a composition comprising a mutant *Listeria monocytogenes* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid, a mutant *Bacillus anthracis* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid, or a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) instructions for the administration of the composition to a host. In some embodiments, the instructions are on a label. In other embodiments, the instructions are on an insert contained within the kit.

[0149] In another aspect, the invention provides a kit comprising both (a) a composition comprising a mutant *Listeria monocytogenes* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid, a mutant *Bacillus anthracis* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid, or a free-living microbe, wherein the nucleic acid of the microbe is modified so that the microbe is attenuated for proliferation; and (b) instructions for selecting a host to which the composition is to be administered. In some embodiments, the instructions are on a label. In other embodiments, the instructions are on an insert contained within the kit.

[0150] In some embodiments of each of the aforementioned aspects, the composition is a vaccine. In some embodiments of each of the aforementioned aspects, the composition is a professional-antigen-presenting cell. In some embodiments of each of the aforementioned aspects, the nucleic acid of the free-living microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid. In some embodiments, the microbe has been s-59/UVA treated. In some embodiments, the microbe is defective with respect to a DNA repair enzyme.

#### Additional embodiments of the invention

[0151] In one embodiment, the invention includes a vaccine composition comprising a free-living microbe in which the microbial nucleic acid is modified so that the proliferation of the microbe is attenuated and/or comprising an antigen-presenting cell that has been antigen-loaded and/or activated or matured through infection with a free-living

microbe in which the microbial nucleic acid is modified so that the proliferation of the microbe is attenuated, wherein the microbial gene expression is substantially unaffected. In one embodiment, the microbial gene expression is substantially unaffected so that an antigen is expressed at a level sufficient to stimulate an immune response upon administration of the microbe to an individual. In one embodiment, the proliferation of the microbe is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the microbe is attenuated by about 0.3 to > 10 log, about 2 to > 10 log, about 4 to > 10 log, about 6 to > 10 log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In one embodiment, the expression of an antigen by the microbe is at least about 10%, about 25%, about 50%, about 75%, or at least about 90% of the expression of the antigen by a microbe in which the microbial nucleic acid is not modified. In one embodiment, the antigen expressed is an antigen from the microbe itself. In one embodiment, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. In one embodiment, the antigen is a disease associated antigen. In one embodiment, the antigen is associated with a disease selected from the group consisting of infectious diseases, autoimmune diseases, allergies, cancers, and other hyperproliferative diseases. In one embodiment, the antigen is a tumor associated antigen. In one embodiment, the tumor antigen is selected from the group consisting of differentiation antigens, tissue-specific antigens, developmental antigens, tumor-associated viral antigens, cancer-testis antigens, embryonic antigens, oncoprotein antigens, over-expressed protein antigens and mutated protein antigens. In one embodiment, the tumor antigen is selected from the group consisting of mesothelin, Sp17, gp100, , PR3, PAGE-4, TARP, WT-1, NY-ESO-1 and SPAS-1. In one embodiment, the microbial nucleic acid is modified by a method selected from the group consisting of exposing the microbe to radiation and reacting the microbe with a nucleic acid targeted compound that causes the modification of the microbial nucleic acid. In a preferred embodiment, the microbial nucleic acid is modified by reacting the microbe with a nucleic acid targeted compound that reacts directly with the nucleic acid. In one embodiment, the nucleic acid targeted compound is targeted to the nucleic acid by a mode selected from the group consisting of intercalation, minor groove binding, major groove binding, electrostatic binding, and sequence-specific binding. In one embodiment, the nucleic acid targeted compound comprises a nucleic acid alkylator. In a preferred embodiment, the nucleic acid targeted compound is  $\beta$ -alanine, N-(acridin-9-

yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In one embodiment, the nucleic acid targeted compound that reacts directly with the nucleic acid reacts upon activation of the compound by irradiation, preferably by UVA irradiation. In one embodiment, the nucleic acid targeted compound activated by UVA irradiation is a psoralen. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the nucleic acid targeted compound indirectly causes the modification of the nucleic acid. In one embodiment, the nucleic acid targeted compound indirectly causes modification upon activation by irradiation, preferably by UVA irradiation. In one embodiment, the microbe comprises a genetic mutation. In one embodiment, the genetic mutation results in the attenuation of the ability of the microbe to repair microbial nucleic acid that has been modified. In one embodiment, the genetic mutation is in the gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the microbe. In one embodiment, the mutation is in one or more of the genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes. In one embodiment, the genetic mutation results in the attenuation in the activity of a DNA repair enzyme selected from the group consisting of PhrB, UvrA, UvrB, UvrC, UvrD and RecA. In a further embodiment, the microbes containing these mutations are modified by reaction with a psoralen activated by UVA irradiation. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the microbe is selected from the group consisting of bacteria, protozoa and fungi. In one embodiment, the microbe is a bacteria. In one embodiment, the bacteria is an intracellular bacteria. In a preferred embodiment, the bacteria is a *Listeria*, preferably *Listeria monocytogenes*. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to invade non-phagocytic cells without significantly affecting the uptake of the *Listeria* by phagocytic cells. In one embodiment, the *Listeria* mutation is in an internalin gene(s). In one embodiment, the *Listeria* mutation is in the gene selected from the group consisting of *inlA*, *inlB*, and any gene encoding an internalin. In one embodiment, the *Listeria monocytogenes* comprises a genetic mutation in both the *inlA* and *inlB* genes. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to escape the phagolysosome of an infected cell. In one embodiment, the *Listeria* mutation is in the *hly* gene. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the polymerization of actin by the *Listeria*. In a preferred embodiment, the *Listeria* mutation is in the *actA* gene. In one

embodiment, the *Listeria* comprises mutations in the *actA* gene and one or more internalin genes. In a preferred embodiment, the *Listeria* comprises a mutation in the *actA* gene and the *inlB* gene, preferably the *Listeria* comprises an *actA/inlB* deletion mutant. In a preferred embodiment, the *Listeria monocytogenes actA/inlB* deletion mutant further comprises a deletion mutation in the *uvrAB* gene.

[0152] In one embodiment, the invention includes a vaccine composition comprising a bacteria in which the bacterial nucleic acid is modified so that the proliferation of the bacteria is attenuated, wherein the bacterial gene expression is substantially unaffected. In one embodiment, the bacterial gene expression is substantially unaffected so that an antigen is expressed at a level sufficient to stimulate an immune response to the bacteria upon administration of the bacteria to an individual. In one embodiment, the proliferation of the bacteria is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the microbe is attenuated by about 0.3 to > 10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In one embodiment, the expression of an antigen by the bacteria is at least about 10%, about 25%, about 50%, about 75%, or at least about 90% of the expression of the antigen by a bacteria in which the bacterial nucleic acid is not modified. In one embodiment, the bacterial nucleic acid is modified by a method selected from the group consisting of exposing the bacteria to radiation and reacting the bacteria with a nucleic acid targeted compound that causes the modification of the bacterial nucleic acid. In a preferred embodiment, the bacterial nucleic acid is modified by reacting the bacteria with a nucleic acid targeted compound that reacts directly with the nucleic acid. In one embodiment, the nucleic acid targeted compound is targeted to the nucleic acid by a mode selected from the group consisting of intercalation, minor groove binding, major groove binding, electrostatic binding, and sequence-specific binding. In one embodiment, the nucleic acid targeted compound comprises a nucleic acid alkylator. In a preferred embodiment, the nucleic acid targeted compound is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In one embodiment, the nucleic acid targeted compound that reacts directly with the nucleic acid reacts upon activation of the compound by irradiation, preferably by UVA irradiation. In one embodiment, the nucleic acid targeted compound activated by UVA irradiation is a psoralen. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the

nucleic acid targeted compound indirectly causes the modification of the nucleic acid. In one embodiment, the nucleic acid targeted compound indirectly causes modification upon activation by irradiation, preferably by UVA irradiation. In one embodiment, the bacteria comprises a genetic mutation. In one embodiment, the genetic mutation results in the attenuation of the ability of the bacteria to repair bacterial nucleic acid that has been modified. In one embodiment, the genetic mutation is in the gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the bacteria. In one embodiment, the mutation is in one or more of the genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes. In one embodiment, the genetic mutation results in the attenuation in the activity of a DNA repair enzyme selected from the group consisting of PhrB, UvrA, UvrB, UvrC, UvrD and RecA. In a preferred embodiment, the bacteria containing these mutations are modified by reaction with a psoralen activated by UVA irradiation. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the bacteria is selected from the group consisting of Gram positive bacteria, Gram negative bacteria, intracellular bacteria and mycobacteria. In one embodiment, the bacteria is selected from the group consisting of *Bacillus anthracis*, *Cholera*, *Bordetella pertussis*, *Corynebacterium diphtheriae*, *E. coli*, *Borrelia burgdorferi* (Lyme), *Streptococcus pneumoniae*, *Salmonella*, *Staphylococcus sp.*, *Mycobacterium tuberculosis*, *Brucella abortus*, *Brucella melitensis*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Yersinia pestis*, *Shigella sp.*, *Francisella tularensis*, and *Streptococcus pyogenes*. In one embodiment, the bacteria is a mycobacteria. In one embodiment, the mycobacteria is *Mycobacterium tuberculosis*. In one embodiment, the *Mycobacterium tuberculosis* comprises a *uvrAB* deletion mutation. In one embodiment, the *Mycobacterium tuberculosis* comprises a conditional *recA* mutation. In one embodiment, the bacteria is an intracellular bacteria. In one embodiment, the intracellular bacteria is *Bacillus anthracis*. In one embodiment, the *Bacillus anthracis* comprises a *uvrAB* deletion mutation. In one embodiment, the *Bacillus anthracis* comprises a conditional *recA* mutation. In one embodiment, the intracellular bacteria is *Yersinia pestis*. In one embodiment, the *Yersinia pestis* comprises a *uvrAB* deletion mutation. In one embodiment, the *Yersinia pestis* comprises a conditional *recA* mutation.

[0153] The invention includes medicaments comprising the above compositions and methods of use of the above compositions, such as vaccination of an individual. In one embodiment, the invention includes a method of using a vaccine of the present invention

comprising administering the vaccine to an individual. In one embodiment, the vaccination is performed by administration of the vaccine by a route selected from the group consisting of oral, nasal, intravenous, intradermal, intraperitoneal, intramuscular, intralymphatic, and subcutaneous. In one embodiment, the vaccine is administered using a prophylactic regimen to an individual having no signs of the disease against which the vaccine is targeted. In one embodiment, the vaccine is administered using a therapeutic regimen to an individual having symptoms of the disease against which the vaccine is targeted. In one embodiment, the vaccine comprises a tumor antigen targeting a cancer and the therapeutic vaccination results in a lessening of the symptoms of the cancer. In one embodiment, the mean tumor volume in a vaccinated individual decreases by at least about 5%, also about 10%, also about 25%, also about 50%, also about 75%, also about 90% or about 100%. In one embodiment, the vaccine is administered to a mouse using either a prophylactic or therapeutic regimen, wherein the mouse is a model system that can be implanted with tumor cells in order to establish tumors in the mice, wherein the vaccine contains at least one antigen of the implanted tumor. The tumors are implanted in the mice either after (prophylactic regimen) or before (therapeutic regimen) the vaccine is administered to the mice. In one embodiment, the mean tumor volumes in mice vaccinated using either a prophylactic or a therapeutic regimen are less than the tumor volumes in similar mice that are either not vaccinated, or are vaccinated with a similar vaccine vehicle that expresses an irrelevant antigen (control mice). In one embodiment, the mean tumor volumes in the vaccinated mice is at least about 5%, about 10%, about 25%, about 50%, about 75%, about 90% or about 100% less than the mean tumor volumes in the control mice. In one embodiment, the median survival time of the mice vaccinated using either a prophylactic or a therapeutic regimen is at least about 2, about 5, about 7 or at least about 10 days longer than in the control mice.

[0154] In one embodiment, the invention includes a method of making a vaccine composition comprising treating a microbial population so that the microbial nucleic acid is modified so that the proliferation of the microbial population is attenuated, wherein the microbial gene expression is substantially unaffected. In another embodiment, the invention includes a method of making a vaccine composition comprising treating a microbial population so that the microbial nucleic acid is modified so that the proliferation of the microbial population is attenuated, wherein the microbial gene expression is substantially unaffected, and then using that microbial population to load an antigen-presenting cell with antigen and induce activation/maturation of the antigen-presenting cell.

In one embodiment, the microbial population is treated by irradiation. In one embodiment, the microbial population is treated by reacting with a nucleic acid targeted compound that indirectly causes the modification of the nucleic acid. In a further embodiment, the nucleic acid targeted compound is activated by irradiation, wherein activation of the compound causes the indirect modification of the nucleic acid. In a further embodiment, activation of the nucleic acid targeted compound results in a reactive oxygen species that modifies the nucleic acid. In one embodiment, the microbial population is treated by reacting with a nucleic acid targeted compound that reacts directly with the nucleic acid. In one embodiment, the nucleic acid targeted compound is reacted at a concentration of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM. In one embodiment, the nucleic acid targeted compound comprises an alkylator. In one embodiment, the alkylator is selected from the group consisting of mustards, mustard intermediates and mustard equivalents. In one embodiment, the nucleic acid targeted compound comprises a nucleic acid targeting group selected from the group consisting of intercalators, minor groove binders, major groove binders, electrostatic binders, and sequence-specific binders. In one embodiment, the nucleic acid targeted compound reacts directly with the nucleic acid upon activation of the compound. In one embodiment, the activation of the compound is by irradiation. In one embodiment, the irradiation is UVA irradiation. In a preferred embodiment, the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation. In one embodiment, the psoralen compound is at a concentration of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, and the UVA irradiation is at a dose of about 0.1 - 100 J/cm<sup>2</sup>, also about 0.1-20 J/cm<sup>2</sup>, or about 0.5-5 J/cm<sup>2</sup> or about 2-4 J/cm<sup>2</sup>. In one embodiment, the proliferation of the microbial population is attenuated by at least about 0.3 log, also at least about 1 log, about 2 log, about 3 log, about 4 log, about 6 log, or at least about 8 log. In another embodiment, the proliferation of the microbial population is attenuated by about 0.3 to > 10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, about 0.3-6 log, about 0.3-5 log, about 1-5 log, or about 2-5 log. In one embodiment, the expression of an antigen by the microbial population is at least about 10%, about 25%, about 50%, about 75%, or at least about 90% of the expression of the antigen by a microbial population that has not been treated to modify the nucleic acid. In one embodiment, the antigen expressed is an antigen from the microbe itself. In one embodiment, the microbe is *Mycobacterium tuberculosis* and the antigen is from *Mycobacterium tuberculosis*. In one embodiment, the microbe is *Bacillus*

*anthracis* and the antigen is from *Bacillus anthracis*. In one embodiment, the microbe comprises a heterologous nucleic acid sequence encoding an antigen. In one embodiment, the antigen is a disease associated antigen. In one embodiment, the antigen is associated with a disease selected from the group consisting of infectious diseases, autoimmune diseases, allergies, cancers, and other hyperproliferative diseases. In one embodiment, the antigen is a tumor associated antigen. In one embodiment, the tumor antigen is selected from the group consisting of differentiation antigens, tissue specific antigens, developmental antigens, tumor-associated viral antigens, cancer-testis antigens, embryonic antigens, oncoprotein antigens, over-expressed protein antigens and mutated protein antigens. In one embodiment, the tumor antigen is selected from the group consisting of mesothelin, Sp17, gp100, PR3, PAGE-4, TARP, WT-1, NY-ESO-1 and SPAS-1. In one embodiment, the microbe comprises a genetic mutation. In one embodiment, the genetic mutation results in the attenuation of the ability of the microbe to repair microbial nucleic acid that has been modified. In one embodiment, the genetic mutation is in the gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes, depending on the genus and species of the microbe. In one embodiment, the genetic mutation is in one or more of the genes selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or their functionally equivalent genes. In one embodiment, the genetic mutation results in the attenuation in the activity of a DNA repair enzyme selected from the group consisting of PhrB, UvrA, UvrB, UvrC, UvrD and RecA. In a further embodiment, microbes having these mutations are treated with a psoralen activated by UVA irradiation. In one embodiment, the microbe is selected from the group consisting of bacteria, protozoa and fungi. In one embodiment, the microbe is a bacteria. In one embodiment, the bacteria is an intracellular bacteria. In a preferred embodiment, the bacteria is a *Listeria*, preferably *Listeria monocytogenes*. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to invade non-phagocytic cells without significantly affecting the uptake of the *Listeria* by phagocytic cells. In one embodiment, the *Listeria* mutation is in an internalin gene(s). In one embodiment, the *Listeria* mutation is in the gene selected from the group consisting of *inlA*, *inlB*, and any gene encoding an internalin. In one embodiment, the *Listeria monocytogenes* comprises a genetic mutation in both the *inlA* and *inlB* genes. In one embodiment, the *Listeria* comprises a mutation that results in the attenuation of the ability of the *Listeria* to escape the phagolysosome of an infected cell. In one embodiment, the *Listeria* mutation is in the *hly* gene. In one embodiment, the *Listeria*



comprises a mutation that results in the attenuation of the polymerization of actin by the *Listeria*. In a preferred embodiment, the *Listeria* mutation is in the *actA* gene. In one embodiment, the *Listeria* comprises mutations in the *actA* gene and one or more internalin genes. In a preferred embodiment, the *Listeria* comprises a mutation in the *actA* gene and the *inlB* gene, preferably the *Listeria* comprises an *actA/inlB* deletion mutant. In a preferred embodiment, the *Listeria monocytogenes actA/inlB* deletion mutant further comprises a deletion mutation in the *uvrAB* gene.

## EXAMPLES

### EXAMPLE 1

Psoralen treatment of *Listeria* strains providing attenuation of proliferation while maintaining expression of OVA antigen.

[0155] Several strains of *Listeria monocytogenes* that have been modified to express ovalbumin, a heterologous chicken OVA antigen, were reacted with 4'-(4-amino-2-oxa)butyl-4,5,8-trimethylpsoralen (S-59 prepared from solid (Ash-Stevens, Riverview, MI) as 3 mM solution by Ben Venue, Cleveland, OH (see US patent 5,399,719)) and UVA light (320-400 nm). The resulting *Listeria* were assayed to assess the reduction in the log titer of viable *Listeria* as well as for the expression of the OVA antigen by the *Listeria*. The *Listeria* strains were provided by Dr. Dan Portnoy at the University of California, Berkeley and modified to contain the OVA antigen as discussed in Example 8. These were DP-L4056 (wild-type), DP-L4029 (10403S  $\Delta$ actA, phage cured deletion mutation in the Act A gene, see Skoble et al., Journal of Cell Biology, 150:527-537 (2000) and Lauer et al., Journal of Bacteriology 184(15):4177-4186 (2002)), DP-L4364 (10403S  $\Delta$ lplA, deletion mutation in phospholipase A gene) and DP-L4017 (10403S hly<sub>L461T</sub>, point mutation in the hemolysin gene, see Glomski et al., Journal of Cell Biology 156(6): 1029-1038, (2002)). The strains were grown in BHI medium (Brain Heart Infusion, Fisher Scientific) at 37 °C at 300 rpm to a concentration of approximately  $1 \times 10^9$  CFU/mL (to an absorbance at 600 nm of 0.5). A 1.0 mL aliquot of each strain was transferred to duplicate 15 mL tubes. Each tube was centrifuged at 4 °C for 20 minutes at 2300 x g, the supernatant removed and 5 mL of PBS (phosphate buffered saline, Hyclone) with 1% BSA with and without the S-59 was added to the duplicate tubes ( $1 \times 10^8$  CFU/mL). The S-59 was added at a concentration of

100 nM. Samples were placed in 6 well culture plates and UVA irradiated at a dose of approximately  $2 \text{ J/cm}^2$  (FX1019 irradiation device, Baxter Fenwal, Round Lake, IL). Each sample was then transferred to a 15 mL tube, centrifuged as above, and the supernatant removed. These were washed with 5 mL of PBS, centrifuged and the supernatant removed and the final bacterial pellet was suspended in 0.5 mL of PBS. A 100  $\mu\text{L}$  sample of each was used to determine the bacterial titer by serial dilution. Each dilution was plated onto an LB (Luria-Bertani, Q-Biogene, Carlsbad, CA) plate and incubated overnight at  $37^\circ\text{C}$  and the colonies were counted to measure the bacterial titer.

[0156] The antigen presentation of the bacterial samples was assessed using a murine DC 2.4 cell line (dendritic cell line from the Dana Farber Cancer Institute, see Shen et al., J Immunol 158(6):2723-30 (1997)) and a B3Z T cell hybridoma (obtained from Dr. Shastri, University of California, Berkeley). The B3Z is a lacZ inducible CD8+ T cell hybridoma that expresses a  $\beta$ -galactosidase gene upon recognition of OVA antigen in context of MHC class I molecules. The metabolism of CPRG (chlorophenolred- $\beta$ -D-galactopyranoside, Calbiochem, La Jolla, CA), a substrate for the  $\beta$ -galactosidase, was used to assess the level of  $\beta$ -galactosidase produced, which is directly correlated to the amount of OVA antigen presented by the DC 2.4 cells. The DC 2.4 cells and the B3Z T cell hybrid were maintained in RPMI 1640 culture medium (RPMI, Invitrogen) with 10% FBS (fetal bovine serum, HyClone). The DC 2.4 cells were transferred in 200  $\mu\text{L}$  aliquots to the wells of a 96 well culture plate ( $1 \times 10^5$  DC 2.4 per well). The bacterial samples were serially diluted 50  $\mu\text{L}$  stock to 450  $\mu\text{L}$  PBS down to  $1 \times 10^5$  CFU/mL (S-59 treated samples are CFU equivalents, i.e. it is the number of colony forming units prior to S-59 treatment). A 20  $\mu\text{L}$  aliquot of each dilution is transferred to a well containing the DC 2.4 cells to give approximately  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ ,  $1 \times 10^7$ , or  $1 \times 10^8$  CFU/mL. In addition, a 20  $\mu\text{L}$  aliquot of PBS only was added as a negative control. The samples were incubated for 1 hour at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The plate was washed three times with PBS to remove extracellular bacteria. A 200  $\mu\text{L}$  aliquot of B3Z T cells ( $1 \times 10^5$  cell) and 100  $\mu\text{g/mL}$  Gentamycin (Sigma) was added to each well. As a positive control, 100 nM SL8 OVA<sub>257-264</sub> peptide (SL8 OVA antigen, SIINFEKL, SEQ ID NO:1, Invitrogen, San Diego, CA) was added to a well containing  $1 \times 10^5$  each of the DC 2.4 and B3Z cells. The samples were incubated overnight at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . The plate was centrifuged for 3 minutes at  $400 \times g$  and each well washed with 250  $\mu\text{L}$  of PBS. A 100  $\mu\text{L}$  aliquot of PBS containing 100  $\mu\text{M}$  2-mercaptoethanol, 9 mM  $\text{MgCl}_2$ , 0.125% Igepal CA-630

((Octaphenoxy)polyethoxyethanol, Sigma), and 0.15 mM CPRG was added to each well. The samples were incubated at 37 °C for at least 4 hours. The absorbance was measured at 595 nm with a reference measurement at 655 nm using a plate reader. The results for the bacterial titer and the antigen presentation of S-59 treated relative to the untreated (100 bacteria per DC 2.4) is given in Table 1. The results indicate that at a level of 100 bacterial cells added per DC 2.4, the antigen presentation is approximately 55-85% of the untreated sample. Since the bacterial titer was reduced by approximately  $10^4$ , this indicates that sufficient antigen presentation is maintained with considerable attenuation of the proliferation of the *Listeria*.

Table 1 Log attenuation and antigen presentation of *Listeria* strains expressing OVA antigen treated with 100 nM psoralen S-59 and 2 J/cm<sup>2</sup> UVA light.

<i>Listeria</i> strain	Log attenuation	% antigen presented*
DP-L4056	4.02	74.6
DP-L4029	4.14	54.9
DP-L4364	4.53	84.3
DP-L4017	4.11	55.2

\* As percent of untreated, measured at 100 *Listeria* per DC 2.4 cell.

[0157] A similar procedure was done using the DP-L4056 wild-type strain. The bacteria was treated with 100, 200, 400, 800 or 1000 nM S-59, the remaining titer determined and the antigen presentation measured as detailed above. The results for the bacterial titer and antigen presentation (100 *Listeria* per DC 2.4 cell) are shown in Table 2 and plotted in Figure 1. This data indicates that the antigen presentation is significant over a broad range of attenuation in the *Listeria* growth, including presentation of antigen with complete inhibition of proliferation (i.e. to the limit of detection).

Table 2 Log attenuation and antigen presentation of *Listeria* strain DP-L4056 expressing OVA antigen treated with varying concentrations of psoralen S-59 and 2 J/cm<sup>2</sup> UVA light.

S-59 concentration (nM)	Log titer	Log attenuation	% antigen presented*
0	8.64	0	-
100	4.34	4.30	75.0

200	3.10	5.54	58.9
400	2.48	6.16	30.3
800	<1	>7.64	23.6
1000	<1	>7.64	5.6

\* As percent of untreated, measured at 100 *Listeria* per DC 2.4 cell.

## EXAMPLE 2

DNA targeted alkylator treatment of *Listeria* strains providing attenuation of proliferation while maintaining expression of OVA antigen.

[0158] A procedure was done similarly to Example 1 only using the compound  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester (Compound 1, ChemSyn, Harrisonville, MO, see US patent 6,093,725). The *Listeria* strains used were DP-L4056 and DP-L4017. Compound 1 (1 mM in acidic BBS (blood bank saline), 135  $\mu$ L of 1.48 M  $H_3PO_4$  per 100 mL BBS) was added to 5 mL of bacteria at  $1 \times 10^8$  CFU/mL to concentrations of 0, 0.5, 1, 2, 5, and 10  $\mu$ M and the samples incubated for 2 hours at room temperature. After the incubation, the bacterial titer and antigen presentation was assessed as per Example 1. For the antigen presentation, the *Listeria* strains were diluted to  $1 \times 10^2$ ,  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ , or  $1 \times 10^7$  CFU/mL. The log titer, log attenuation and antigen presentation as a percent of untreated (1 *Listeria* per DC 2.4) as a function of compound 1 concentration is given in Table 3 and Figures 2A,B. The results indicate that compound 1 is also effective, e.g. at 1  $\mu$ M, at providing sufficient antigen presentation with considerable attenuation of the proliferation of the *Listeria*.

Table 3 Log attenuation and antigen presentation of *Listeria* strains treated with varying concentrations of compound 1.

[compound 1] $\mu$ M	Log attenuation		% antigen presented*	
	DP-L4056	DP-L4017	DP-L4056	DP-L4017
0.5	1.04	1.02	56.6	70.0
1	3.47	3.43	35.0	43.0
2	6.47	6.52	18.5	25.4
5	>7.0	>7.0	3.7	2.0
10	>7.0	>7.0	Not measured	Not measured

\* As percent of untreated, measured at 1 *Listeria* per DC 2.4 cell.

### EXAMPLE 3

Comparison of attenuation of proliferation by psoralen treatment of *uvrAB* mutant vs. wild-type *Escherichia coli*.

[0159] The psoralen treatment of a mutant *Escherichia coli* (*E. coli*) strain that is deficient in the ability to repair nucleic acid damage was compared to a wild-type strain. *E. coli* strains AB1157 (wild-type) and CSR 603 (*uvrA*, *recA*, *phr* mutant obtained from Dr. Aziz Sancar, University of North Carolina, see Harm, Mutation Research 60:263-270 (1979)). This example compares the attenuation of AB1157 vs mutant CSR603 grown in 3 mL of LB media with streptomycin overnight at 37 °C on an orbital shaker at 250 rpm. A 2 mL aliquot of this was added to 100 mL of LB media at 30 °C and placed on the shaker for approximately 5 hours, until the absorbance at 600 nm was 0.9 OD, approximately  $1 \times 10^9$  CFU/mL. For each strain, approximately 0.5 mL of the bacterial stock was added to a 15 mL tube and centrifuged at 4 °C for 20 minutes at 2300 x g. The supernatant was removed and each pellet was suspended in 5 mL of PBS containing 0, 1, 10, 100, and 1000 nM of psoralen S-59. Each sample was transferred to a 6 well culture plate and irradiated as per Example 1. The samples were serially diluted and the titer determined as per Example 1. The results are shown in Table 4 and Figure 3. The results indicate that psoralen treatment of the *uvrABC* mutant results in greater attenuation in the proliferation of the bacteria (lower titer remaining) for a given psoralen concentration.

Table 4 Attenuation of E coli wild-type vs. *uvrABC* mutant with psoralen treatment.

[S-59] nM	Bacterial log titer		Log attenuation	
	Wild-type	<i>uvrABC</i> mutant	Wild-type	<i>uvrABC</i> mutant
0	8.0	7.75	-	-
1	8.08	5.52	0	2.23
10	7.99	4.68	0.01	3.07
100	7.57	3.1	0.43	4.9
1000	4.91	<1	3.09	>6.65

## EXAMPLE 4

Therapeutic vaccination of mice using *Listeria* strains with and without S-59 treatment.

[0160] In order to assess the utility of S-59 treated *Listeria* as a vaccine, a C57Bl/6 mouse melanoma tumor model was used. C57Bl/6 mice (Charles River, Hollister, CA) were shaved and implanted subcutaneously with  $2 \times 10^5$  B16.F10.Mo520.10 cells (B16-OVA expressing melanoma cells obtained from Dr. Kenneth Rock, University of Massachusetts, see Mandl et al., Proc Natl Acad Sci USA 95:8216 (1998)) in 100  $\mu$ L of HBSS. *Listeria monocytogenes* strains DP-L4056 and DP-L4017 containing the OVA antigen were prepared with or without S-59 treatment (20 nM S-59 UVA dosed as per Example 1). In addition, the wild-type strain DP-L4056 without the OVA antigen was used as a control. The log titer of the S-59 treated samples was determined to assess the log attenuation due to the psoralen treatment (Table 6). The *Listeria* were suspended in HBSS (Hanks Balanced Salt Medium, Gibco) and groups of 10-12 mice were vaccinated three times with a 100  $\mu$ L intraperitoneal injection of each strain, as well as a group injected with HBSS vehicle. The vaccination dose (total CFU per vaccination) for the various strains is indicated in Table 6. The doses corresponded to 0.1 LD<sub>50</sub> for the non S-59 treated *Listeria* and the maximum possible dose for the S-59 treated *Listeria*. The vaccination was done at 3, 10 and 17 days after tumor implant. The mice were observed for palpable tumors. Once observed, the opposing diameters of the tumors were measured twice a week. If the tumor measured 20 mm in any direction, the mice were sacrificed. The mean tumor volume as a function of days post B16-OVA implant are shown in Figure 4 and Table 5. The percent survival of mice per group is plotted in Figure 5 and the median survival is given in Table 6. This example shows that high doses of S-59 treated *Listeria* strains can be safely given to mice, resulting in a good anti tumor response.

Table 5 Tumor volume at days post implant for mice implanted with B16-OVA and vaccinated with the identified *Listeria* strains.

Vaccine sample	Mean tumor volume (mm <sup>2</sup> )				
	Day 10	Day 13	Day 17	Day 20	Day 24
HBSS	48.2	158.8	515.1	1603	2444
DP-L4056	35.3	123.6	571.8	1304	2123
DP-L4056-OVA	34.6	31.8	36.6	101.3	404.8

DP-L4056-OVA + S-59	18.9	14.6	14.9	46.2	210.4
DP-L4017-OVA	22.7	26.8	73.8	164.6	689.5
DP-L4017-OVA + S-59	33.5	56.7	79.3	146.3	464.0

Table 6 Vaccination dose and median survival for *Listeria* strains with and without 20 nM S-59 treatment (2 J/cm<sup>2</sup> UVA).

Vaccination sample	Dose (CFU)	Median survival (days)	Log titer reduction
HBSS	-	20	-
DP-L4056	5 x 10 <sup>3</sup>	22.5	-
DP-L4056-OVA	2 x 10 <sup>5</sup>	30	-
DP-L4056-OVA + S-59	1 x 10 <sup>8</sup>	30	3.76*
DP-L4017-OVA	1 x 10 <sup>7</sup>	30	-
DP-L4017-OVA + S-59	1 x 10 <sup>8</sup>	32	4.27*

\* Value is average of three preparations.

#### EXAMPLE 5

Assessment of antigen-specific immune responses after vaccination.

[0161] The vaccines of the present invention can be assessed using a variety of *in vitro* and *in vivo* methods. These methods are exemplified using a *Listeria* based vaccine but can be used to evaluate the potential efficacy of any microbial based vaccine of the present invention.

[0162] Some assays involve the analysis of antigen-specific T cells from the spleens of mice that have been vaccinated. C57Bl/6 mice are vaccinated, for example intraperitoneal injection of 0.1 LD<sub>50</sub>, with a *Listeria*-OVA strain, where the *Listeria* may be treated to attenuate proliferation (e.g. S-59 treatment). Seven days after the vaccination, the spleen cells of the mice are harvested (typically 3 mice per group) by placing the spleens into ice cooled RPMI 1640 medium and preparing a single cell suspension from this. As an alternative, the lymph nodes of the mice could be similarly harvested, prepared as a single cell suspension and substituted for the spleen cells in the assays described below. Typically, spleen cells are assessed for intravenous or intraperitoneal

administration of the vaccine while spleen cells and cells from lymph nodes are assessed for intramuscular, subcutaneous or intradermal administration of the vaccine.

[0163] Unless otherwise noted, all antibodies used in these examples can be obtained from Pharmingen, San Diego, CA.

[0164] ELISPOT Assay:

[0165] A *Listeria* strain having an OVA antigen is assessed for the quantitative frequency of antigen-specific T cells generated upon immunization in a mouse model using an ELISPOT assay. The antigen-specific T cells evaluated are OVA specific CD8+ or LLO specific CD8+ or CD4+ T cells. This OVA antigen model assesses the immune response to a heterologous tumor antigen inserted into the vaccine and could be substituted with any antigen of interest. The LLO antigen is specific to *Listeria*, and could be substituted for an appropriate antigen for any microbial vector used as the vaccine vehicle. The specific T cells are assessed by detection of cytokine release (e.g. IFN- $\gamma$ ) upon recognition of the specific antigen. PVDF-based 96 well plates (BD Biosciences, San Jose, CA) are coated overnight at 4°C with an anti-murine IFN- $\gamma$  monoclonal antibody (mAb R4; 5  $\mu$ g/mL). The plates are washed and blocked for 2 hours at room temperature with 200  $\mu$ L of complete RPMI. Spleen cells from vaccinated mice (or non vaccinated control mice) are added at  $2 \times 10^5$  cells per well and incubated for 20 to 22 hours at 37°C in the presence of various concentrations of peptides ranging from about 0.01 to 10  $\mu$ M. The peptides used are either SL8, an MHC class I epitope for OVA, LLO<sub>190</sub> (NEKYAQAYPNVS, SEQ ID NO:2, Invitrogen) an MHC class II epitope for listeriolysin O (*Listeria* antigen), or LLO<sub>296</sub> (VAYGRQVYL, SEQ ID NO:3), an MHC class I epitope for listeriolysin O. After washing, the plates are incubated with secondary biotinylated antibodies specific for IFN- $\gamma$  (XMG1.2) diluted in PBS to 0.5  $\mu$ g/mL. After incubation at room temperature for 2 hours, the plates are washed and incubated for 1 hour at 37 °C with a 1 nm gold goat anti-biotin conjugate (GAB-1; 1:200 dilution; Ted Pella, Redding, CA) diluted in PBS containing 1 % BSA. After thorough washing, the plates are incubated at room temperature for 2 to 10 minutes with substrate (Silver Enhancing Kit; 30  $\mu$ L/well; Ted Pella) for spot development. The plates are then rinsed with distilled water to stop the substrate reaction. After the plates have been air-dried, spots in each well are counted using an automated ELISPOT plate reader (CTL, Cleveland, OH). The cytokine response is expressed as the number of IFN- $\gamma$  spot-forming cells (SFCs) per  $10^6$  spleen cells for either the OVA specific T cells or the *Listeria* specific T cells.



#### Intracellular Cytokine Staining Assay (ICS):

[0166] In order to further assess the number of antigen-specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells and correlate the results with those obtained from ELISPOT assays, ICS is performed and the cells evaluated by flow cytometry analysis. Spleen cells from vaccinated and control groups of mice are incubated with SL8 (stimulates OVA specific CD8<sup>+</sup> cells) or LLO<sub>190</sub> (stimulates LLO specific CD4<sup>+</sup> cells) for 5 hours in the presence of Brefeldin A (Pharmingen). The Brefeldin A inhibits secretion of the cytokines produced upon stimulation of the T cells. Spleen cells incubated with an irrelevant MHC class I peptide are used as controls. PMA (phorbol-12-myristate-13-acetate, Sigma) 20 ng/mL and ionomycin (Sigma) 2 µg/mL stimulated spleen cells are used as a positive control for IFN-γ and TNF-α intracellular cytokine staining. For detection of cytoplasmic cytokine expression, cells are stained with FITC-anti-CD4 mAb (RM 4-5) and PerCP-anti-CD8 mAb (53-6.7), fixed and permeabilized with Cytofix/CytoPerm solution (Pharmingen), and stained with PE-conjugated anti-TNF-α mAb (MP6-XT22) and APC-conjugated anti-IFN-γ mAb (XMG1.2) for 30 minutes on ice. The percentage of cells expressing intracellular IFN-γ and/or TNF-α was determined by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA) and data analyzed using CELLQuest software (Becton Dickinson Immunocytometry System). As the fluorescent labels on the various antibodies can all be distinguished by the FACScalibur, the appropriate cells are identified by gating for those CD8<sup>+</sup> and CD4<sup>+</sup> that are stained with either or both of the anti-IFN-γ or anti-TNF-α. This method can also be used to determine the immunogenicity of microbial vaccines, wherein a dendritic cell population, or another antigen presenting cell such as a macrophage population, is incubated with the microbial vector. The resulting antigen presenting cells are injected into the feet of the mice and the cell population from the lymph nodes is assessed for T cells as above.

#### Cytokine Expression of Stimulated Spleen Cells:

[0167] The level of cytokine secretion by the spleen cells of mice can also be assessed for control and vaccinated C57Bl/6 mice. Spleen cells are stimulated for 24 hours with SL8 or LLO<sub>190</sub>. Stimulation with irrelevant peptide HSV-gB<sup>2</sup> (Invitrogen, SSIEFARL, SEQ ID NO:4) is used as a control. The supernatants of the stimulated cells are collected and the levels of T helper-1 and T helper 2 cytokines are determined using an ELISA assay (eBiosciences, CO) or a Cytometric Bead Array Kit (Pharmingen).

[0168] Assessment of Cytotoxic T cell Activity:

[0169] The OVA specific CD8+ T cells can be further evaluated by assessing their cytotoxic activity, either *in vitro* or directly in C57Bl/6 mouse *in vivo*. The CD8+ T cells recognize and lyse their respective target cells in an antigen-specific manner. *In vitro* cytotoxicity is determined using a chromium release assay. Spleen cells of naïve and *Listeria*-OVA (internal) vaccinated mice are stimulated at a 10:1 ratio with either irradiated EG7.OVA cells (EL-4 tumor cell line transfected to express OVA, ATCC, Manassas, VA) or with 100 nM SL8, in order to expand the OVA specific T cells in the spleen cell population. After 7 days of culture, the cytotoxic activity of the effector cells is determined in a standard 4-hour <sup>51</sup>Cr-release assay using EG7.OVA or SL8 pulsed EL-4 cells (ATCC, Manassas, VA) as target cells and EL-4 cells alone as negative control. The YAC-1 cell line (ATCC, Manassas, VA) is used as targets to determine NK cell activity, in order to distinguish the activity due to T cells from that due to NK cells. The percentage of specific cytotoxicity is calculated as  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$ . Spontaneous release is determined by incubation of target cells without effector cells. Maximal release is determined by lysing cells with 0.1% Triton X-100. Experiments are considered valid for analysis if spontaneous release is < 20% of maximal release.

[0170] For the assessment of cytotoxic activity of OVA-specific CD8+ T cells *in vivo*, spleen cells from naïve C57Bl/6 mice are split into two equivalent aliquots. Each group is pulsed with a specific peptide, either target (SL8) or control (HSV-gB<sup>2</sup>), at 0.5 µg/mL for 90 minutes at 37 °C. Cells are then washed 3 times in medium, and twice in PBS + 0.1% BSA. Cells are resuspended at  $1 \times 10^7$  per mL in warm PBS + 0.1% BSA (10 mL or less) for labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR). To the target cell suspension, 1.25 µL of a 5mM stock of CFSE is added and the sample mixed by vortexing. To the control cell suspension, a ten-fold dilution of the CFSE stock is added and the sample mixed by vortexing. The cells are incubated at 37 °C for 10 minutes. Staining is stopped by addition of a large volume (>40 mL) of ice-cold PBS. The cells are washed twice at room temperature with PBS, then resuspended and counted. Each cell suspension is diluted to  $50 \times 10^6$  per mL, and 100 µL of each population is mixed and injected via the tail vein of either naïve or vaccinated mice. After 12-24 hours, the spleens are harvested and a total of  $5 \times 10^6$  cells are analyzed by flow cytometry. The high (target) and low (control) fluorescent peaks are enumerated, and

the ratio of the two is used to establish the percentage of target cell lysis. The *in vivo* cytotoxicity assay permits the assessment of lytic activity of antigen-specific T cells without the need of *in vitro* re-stimulation. Furthermore, this assay assesses the T cell function in their native environment.

#### EXAMPLE 6

ELISPOT and ICS analysis of spleen cells from mice vaccinated with *Listeria* DP-L4056 with and without S-59 treatment.

[0171] *Listeria* strain DP-L4056 with or without the OVA antigen was prepared with or without S-59 treatment and used to vaccinate C57Bl/6 mice as per Example 4 (HBSS control as well), with the exception that administration was intravenous. The vaccination was done on naïve mice at the doses indicated in Tables 7 and 8. The spleens were harvested at 12 days post vaccination. The spleens were assessed by ICS and ELISPOT assays as per Example 5. In addition, the LD<sub>50</sub> was assessed for these *Listeria*. The ICS assay results for both LLO<sub>190</sub> specific CD4<sup>+</sup> T cells and OVA specific CD8<sup>+</sup> T cells, in terms of percent of cells positive for both TNF- $\alpha$  and IFN- $\gamma$ , are given in Table 7 and Figures 6A,B. The ELISPOT assays, in terms of IFN- $\gamma$  SFC per  $2 \times 10^5$  spleen cells is given in table 8 and Figure 7. These results indicate that the S-59 treated sample with OVA stimulates an OVA specific response when dosed at 100-fold excess of the non S-59 treated sample. While the positive OVA specific response is not observed at lower doses, this still provides an increased safety margin as the S-59 treated sample was attenuated by 4 log. In addition, the LD<sub>50</sub> was 10<sup>3</sup>-fold higher for the S-59 treated relative to the untreated sample, indicating that even dosing at 100-fold higher levels, there is a 10-fold level of safety relative to the untreated *Listeria*.

Table 7 Percent of spleen cells that are both TNF- $\alpha$  and IFN- $\gamma$  positive for mice vaccinated with DP-L4056 with or without OVA, with or without S-59 treatment.

Vaccine sample	S-59 treatment	Vaccination dose	% TNF- $\alpha$ /IFN- $\gamma$ positive	
			LLO	OVA
HBSS	No		0.00	0.02
DP-L4056	No	$1 \times 10^5$	1.49	0.01
DP-L4056	Yes	$1 \times 10^5$	0.63	0.02

DP-L4056-OVA	No	$1 \times 10^5$	1.78	1.79
DP-L4056-OVA	Yes	$1 \times 10^5$	0.02	0.02
DP-L4056-OVA	Yes	$1 \times 10^6$	0.06	0.08
DP-L4056-OVA	Yes	$1 \times 10^7$	0.19	0.83
DP-L4056-OVA	Yes	$1 \times 10^8$	0.14	0.50

Table 8 IFN- $\gamma$  SFC per  $10^6$  spleen cells for mice vaccinated with DP-L4-056 with or without OVA, with or without S-59 treatment.

Vaccine sample	Dose	SCF per $2 \times 10^5$ spleen cells for indicated peptide			
		Control	SL8	LLO <sub>190</sub>	LLO <sub>296</sub>
HBSS		3	4	3	3
DP-L4056	$1 \times 10^5$	6	7	176	31
DP-L4056 +S-59	$1 \times 10^5$	5	3	104	87
DP-L4056-OVA	$1 \times 10^5$	11	292	238	31
DP-L4056-OVA +S-59	$1 \times 10^5$	3	8	9	7
DP-L4056-OVA +S-59	$1 \times 10^6$	4	7	10	4
DP-L4056-OVA +S-59	$1 \times 10^7$	4	172	59	11
DP-L4056-OVA +S-59	$1 \times 10^8$	10	171	97	24

#### EXAMPLE 7

Construction of pKSV7-dlBsrFI *uvrAB* for deletion of *uvrAB* from *Listeria* by allelic exchange.

[0172] A mutant strain of *Listeria* unable to repair damage to DNA induced by treatment with psoralen and UVA light was created by substantially deleting the ultraviolet light resistance (*uvr*) AB gene (*uvrAB*) in *Listeria*. These mutants are known as DNA repair mutants, or alternatively, nucleotide excision repair (NER) mutants. Deletion of *uvrAB* from *Listeria* was accomplished by allelic exchange [Camilli et al., Molecular Microbiology 8:143-147 (1993)]. As an example that *uvrAB* could be deleted from any *Listeria* strain, *uvrAB* was deleted from the *Listeria monocytogenes* strains shown in Table 9.

Table 9. Parent *Listeria monocytogenes* strains used for deletion of *uvrAB* by allelic exchange.

<i>Listeria</i> strain	Genotype	Reference
DP-L4056	10403S wild-type, phage cured	Lauer et. al., J. Bacteriol. 184:4177-4186 (2002).
DP-L4017	10403S, L461T LLO	Glomski et. al., J. Cell Biol. 156:1029-1038 (2001).
DP-L4029	10403S $\Delta$ actA, phage cured	Lauer et. al., J. Bacteriol. 184:4177-4186 (2002); Skoble et. al., J Cell Biol. 150:527-38 (2000).

[0173] The *uvrA* and *uvrB* genes encode 2 of the 3 proteins of the ABC excinuclease complex required for nucleotide-excision repair (NER) in *Listeria* and other bacterial strains of DNA damage inflicted by UV and other agents. The *uvrA* and *uvrB* genes comprise the same operon in the *Listeria* genome, and were thus deleted together in the *Listeria* strains shown in Table 9. The *uvrA* gene maps from *Listeria* nts. 2562547 to 2565461 (SEQ ID NO:5), and the *uvrB* gene maps from *Listeria* nts. 2565469 to 2567459 (SEQ ID NO:6) [Glaser et. al., Science 294:849-852 (2001)]. To delete *uvrAB* by allelic exchange, the *uvrAB* gene was first amplified by PCR, using forward and reverse primers that were approximately 900 base pairs (bps) upstream and downstream, respectively, of *uvrAB*. The *Listeria uvrAB* amplicon was generated using PCR primers Lm-2561677F (SEQ ID NO:7) and Lm-2568330R (SEQ ID NO:8) and DP-L4056 as template, and was 6654 base bps long, encompassing *Listeria* nts. 2561677-2568330 (SEQ ID NO:9). *Listeria* wild-type strain DP-L4056 was cultured overnight at 30 °C in Brain Heart Infusion broth (BHI, Difco), and 10  $\mu$ L of a washed bacterial suspension (prepared by centrifugation of the 3 ml overnight culture, re-suspension of the bacterial pellet in 5 ml PBS, re-centrifugation, and followed by a final re-suspension of the *Listeria* pellet in 1 ml of PBS), was added to a PCR reaction having a final volume of 100  $\mu$ L, that also contained 0.2  $\mu$ M each of Lm-2561677F and Lm-2568330R primers, 2  $\mu$ L pf Vent DNA polymerase (New England Biolabs), together with deoxynucleotide triphosphates, buffer, and MgSO<sub>4</sub> according to the recommendations of the supplier. Successful PCR was confirmed by 0.8% agarose gel electrophoresis in TAE buffer, as demonstrated by the presence of a distinct 6654 bp band following staining with ethidium bromide and visualization by illumination with UV light. The amplicon product was purified from the PCR reaction

using GeneClean (Qbiogene, Carlsbad, CA), in a final volume of 50  $\mu$ L. Subsequently, the amplicon was inserted into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), using 5  $\mu$ L of the purified *uvrAB* amplicon in the ligation mixture. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The correct construction of the pCR2.1-TOPO-*uvrAB* plasmid was verified by digestion with BsrFI (New England Biolabs), followed by 1% agarose/TAE electrophoresis, yielding fragments of 4612, 1388, 1094, 181, 886 and 2424 base pairs.

[0174] The pCR2.1-TOPO-*uvrAB* plasmid was used subsequently to generate a plasmid for allelic exchange, in which nts 2562709 to 2567320 of *uvrAB* (4612 bps) were deleted. All restriction enzymes and T4 DNA ligase for recombinant plasmid construction were obtained from New England Biolabs. To accomplish the deletion of *uvrAB* sequence, one aliquot of the pCR-TOPO/*uvrAB* plasmid (approximately 2  $\mu$ g) was digested with HindIII, BsrFI, and BglII and the 1092 base pair fragment was purified by 1% agarose/TAE gel electrophoresis and GeneClean. In parallel, a second aliquot (approximately 2  $\mu$ g) was digested with XhoI, BsrFI, and BglII enzymes, and the 1050 base pair fragment was purified by 1% agarose/TAE gel electrophoresis and GeneClean. The two 1092 bp and 1050 bp fragments containing compatible BsrFI ends were ligated together and the 2142 bp ligation product was purified using GeneClean. One portion of the 2142 bp ligation product was digested with PstI and the 1486 bp fragment was purified by 1% agarose/TAE gel electrophoresis and GeneClean. A second portion of the 2142 bp ligation product was digested with KpnI and PstI, and the 622 bp fragment was purified by 1% agarose/TAE gel electrophoresis and GeneClean. The parent plasmid vector for allelic exchange, pKSV7 [Camilli et al., Molecular Microbiology 8:143-147 (1993)], was digested with KpnI and PstI and treated with Calf Intestinal Alkaline Phosphatase (CIAP, New England Biolabs) and the 622 bp fragment having KpnI and PstI compatible ends was inserted into the pKSV7 plasmid vector to give pKSV7-K/P-338. Subsequently, the 1486 bp fragment having PstI compatible ends was inserted into the vector construct pKSV7-K/P-338 that was digested with PstI and treated with CIAP. Insertion of the 1486 bp construct in the correct orientation was determined by digestion with KpnI and HindIII to yield fragment sizes of 1253 bp, 865 bp, and 6.9 kb. This plasmid construct is known as pKSV7-dlBsrFI *uvrAB*. The *Listeria* dlBsrFI *uvrAB* portion of the pKSV7 recombinant plasmid was sequenced to verify the fidelity of the *Listeria* sequence and the precise deletion in the *uvrAB* gene of nts. 2562709 to 2567320 (deletion from *uvr* coordinates nts. 2562547 to

2567459). The deleted region is SEQ ID NO:10, with the amp<sup>r</sup>l icon sequence remaining given in SEQ ID NO:11.

[0175] The *uvrAB* genes of *Listeria* strains DP-L4056, DP-L4017, and DP-L4029 (Table 9) were deleted by allelic exchange using plasmid pKSV7-dlBsrFI *uvrAB*, as described previously [Camilli et al., Molecular Microbiology 8:143-147 (1993)]. The plasmid pKSV7-dlBsrFI *uvrAB* was introduced into *Listeria* strains DP-L4056, DP-L4017, and DP-L4029 by electroporation. The *Listeria* strains were made competent for electroporation by first growing a 10 mL overnight culture from an isolated bacterial colony in BHI with shaking at 37°C. A log-phase culture of each strain was then derived by inoculating 2 mL of the overnight culture into 100 mL 0.5M sucrose/BHI (sterile filtered) in a 250 mL flask. The culture was grown to mid-log phase by shaking at 37°C for 2-3 hours until a bacterial density of OD<sub>600</sub>=0.2 was reached. Subsequently, the culture was treated to generate bacteria lacking the peptidoglycan cell wall, known as spheroplasts. 100 µL of a penicillin G stock solution (10 mg/mL, sterile filtered) was added to the mid-log phase culture, followed by shaking at 37°C for 2 hours. The spheroplast culture was pelleted in a 100 mL centrifuge bottle, resuspended with 45 mL of an ice-cold HEPES/sucrose stock solution (1mM HEPES, pH 7.0 / 0.5M sucrose, sterile filtered), and pelleted again by centrifugation. The bacterial pellet was resuspended with 20 mL of HEPES/sucrose, transferred to a 40 mL centrifuge tube, pelleted, and resuspended in 10 mL HEPES/sucrose. 100 µL of a 10 mg/mL lysozyme stock was then added to the bacterial solution, mixed thoroughly, and the culture was incubated 15 minutes at 37 °C without shaking, but was inverted gently twice at 5 minute intervals. The lysozyme-treated culture was then centrifuged at 5000 rpm (3000xg) for 10 min. at 4°C, and resuspended in 10 mL HEPES/sucrose; this process was repeated twice, with careful and thorough resuspension each time. The final step to yield electrocompetent *Listeria* was to resuspend the bacterial pellet in 500 µL HEPES/sucrose.

[0176] For electroporation, 2 µg of pKSV7-dlBsrFI *uvrAB* plasmid DNA was added to 10 µL of DP-L4056, DP-L4017, and DP-L4029 electrocompetent *Listeria*, and the solution was added to a 0.1 cm cuvette. The cuvettes were placed in the electroporation device that was set at 1 KV, 400 ohms, and 25 µFD, and then pulsed. This typically resulted in a time constant of about 5 milli-seconds. The cells were immediately added to 1 mL BHI/sucrose media that was pre-warmed to 30°C, and then incubated for 1 hour at 30°C, without shaking. Following the incubation period, the bacteria were pelleted,

resuspended in 200 ml of BHI broth, and the suspension was plated on BHI-agar containing 10 µg/mL chloramphenicol (BHI/CM10). The plates were then incubated overnight at 30°C, after which colonies corresponding to pKSV7-dlBsrFI *uvrAB* plasmid transformants were visible. The plasmid pKSV7 contains a temperature-sensitive *Listeria* replicon, and thus the plate must be incubated at 30°C in order to visualize chloramphenicol-resistant colonies.

[0177] Allelic exchange of the native *uvrAB* gene in *Listeria* strains DP-L4056, DP-L4017, and DP-L4029 transformed by electroporation with pKSV7-dlBsrFI *uvrAB* plasmid containing a 4612 bp deletion in *uvrAB* was accomplished in two steps comprised of plasmid integration, followed by plasmid excision (including native *uvrAB*) and curing, as described previously [Camilli et al., Molecular Microbiology 8:143-147 (1993)]. Two isolated chloramphenicol-resistant colonies resulting from each of the *Listeria* strains DP-L4056, DP-L4017, and DP-L4029 electroporated with pKSV7-dlBsrFI *uvrAB* plasmid DNA were selected and then each selected colony was streaked onto fresh BHI/CM10 plates, and incubated overnight at 30°C. The next day, a colony was selected from each plate and used to inoculate 10 mL of BHI/CM10 contained in a 250 mL flask, which was then incubated overnight at 30°C with shaking. 10 µL of each of the overnight cultures were then used to inoculate 10 mL of fresh BHI/CM10 media (1:1000 dilution), which were then grown at 41°C with shaking until the cultures reached stationary phase. Following sampling of 10 µL, a plasmid preparation was performed with the remaining overnight *Listeria* culture to insure the presence of the pKSV7-dlBsrFI *uvrAB* plasmid DNA. Once at stationary phase, 10 µL of the cultures were used to inoculate 10 mL of fresh BHI/CM10 media, which was pre-warmed to 41°C, and then incubated overnight at 41°C, with shaking. A sample was then taken from each 41°C overnight culture, and used to streak for isolated colonies on BHI/CM10 plates, that were pre-warmed at 41°C. As the plasmid pKSV7 contains a temperature-sensitive *Listeria* replicon, incubation at 41°C selects for colonies arising from integration through homologous recombination of the pKSV7-dlBsrFI *uvrAB* plasmid with the native *uvrAB* gene in the *Listeria* genome, and thus amplification and expression of the chloramphenicol drug-resistance marker through bacterial cell growth and division. At this point, the *Listeria* strains are merodiploid for the *uvrAB* gene, comprised of the native *uvrAB* gene and the 4612 bp deleted *uvrAB* gene, arising from integration by homologous recombination of the pKSV7-dlBsrFI *uvrAB* plasmid.



[0178] For excision and curing of the native *uvrAB* gene with the pKSV7 plasmid, a single colony was selected from each of the BHI/CM10 plates incubated at 41°C from the step above, and used to inoculate 10 ml of BHI media without chloramphenicol, and then incubated at 30°C overnight with shaking. The cultures were then diluted 1:1000 in 10 mL of fresh BHI media without chloramphenicol, and then incubated at 30°C overnight with shaking for 6 hours. This step was repeated twice. Upon passage of the merodiploid intermediate strains for several generations without drug-selective pressure at a temperature permissive for the pKSV7 plasmid replicon, spontaneous excision of the integrated pKSV7 plasmid occurs, and eventual curing of the plasmid from the bacterium previously. [Camilli et al., Molecular Microbiology 8:143-147 (1993)]. Following the third 1:1000 dilution and 6-hour incubation period, a sample from each culture was taken, and used to streak for isolated colonies on BHI plates (without chloramphenicol), and incubated overnight at 37°C. One hundred isolated colonies were selected from each BHI plate with a toothpick, and a 5 mm-long streak was made with each colony first on a BHI/CM10 plate, followed by a BHI plate. Plates were marked using a grid so that each matched pair of BHI/CM10 and BHI plates were replicates. The BHI/CM10 and BHI replicate plates were incubated overnight at 37°C. Approximately 5% of the colonies replica-plated on BHI/CM10 and BHI plates originating from the two chloramphenicol-resistant colonies from electroporation with DP-L4056, DP-L4017, or DP-L4029 with pKSV7-dlBsrFI *uvrAB* plasmid DNA were drug sensitive (i.e., growth only on BHI plates). These drug-sensitive colonies represented candidates containing the 4612 bp deletion in *uvrAB*. Each of the drug-sensitive colonies were re-streaked for isolated colonies on both BHI/CM10 and BHI plates and incubated overnight at 37°C, to ensure that candidate clones were both pure and drug sensitive. The chloramphenicol-sensitive clones were subjected to PCR using primers Lm-2561677F and Lm-2568330R (*iBid*), in order to identify clones that also contained the *uvrAB* deletion. The amplicon size of clones with the native *uvrAB* gene was 6654 bps, and the amplicon size of the deleted *uvrAB* gene was 2042 bps; about 50% of the chloramphenicol-sensitive clones also contained the deleted *uvrAB* gene. Two clones of the *uvrAB* deleted strains derived from DP-L4056, DP-L4017, and DP-L4029 were selected for further characterization. Glycerol stocks (30 °C overnight culture diluted 1: 1 with sterile LB/40% glycerol) were made for each *uvrAB* mutant strain and stored at -80 °C. These strains are known as shown in Table 10. DP-L4029*uvrAB* (*actA/uvrAB*) was deposited with ATCC on October 3, 2003, assigned PTA-5563.

Table 10. *uvrAB* mutant *Listeria* strains generated by allelic exchange

<i>uvrAB</i> mutant <i>Listeria</i> strain	Parent <i>Listeria</i> Strain
L4056/ <i>uvrAB</i> clone 1	DP-L4056
L4056/ <i>uvrAB</i> clone 2	DP-L4056
L4017/ <i>uvrAB</i> clone 1	DP-L4017
L4017/ <i>uvrAB</i> clone 2	DP-L4017
L4029/ <i>uvrAB</i> clone 1	DP-L4029
L4029/ <i>uvrAB</i> clone 2	DP-L4029

[0179] To demonstrate the increased sensitivity to attenuation with S-59 psoralen and UVA light, a preparation of  $1 \times 10^9$  CFU of the *uvrAB* mutant *Listeria* strains shown in Table 10 were treated either with 2, 20, 100 and 500 nM S-59 (both clones of L4017/*uvrAB* and L4056/*uvrAB*) or 2, 10, 20, and 100 nM S-59 (clone 1 of L4017/*uvrAB* and both clones of L4029/*uvrAB*), UVA irradiated at a dose of 6 J/cm<sup>2</sup> (FX1019), and tested for viability by plating dilutions on BHI plates, exactly as described in Example 1. The results of this study are shown in Table 11A-B (log attenuation as a function of S-59 dose) and Figure 8A-B (log titer remaining as a function of S-59 dose). The results demonstrate clearly that the DNA NER repair mutant strains shown in Table 10 were dramatically more susceptible to photochemical attenuation with psoralen and UVA irradiation, as compared to the parent strains. This data provides unequivocal evidence that significantly and substantially lower levels of S-59 psoralen can be used to inactivate *uvrAB* mutant bacteria to the same extent, as compared to their isogenic counterpart.

Table 11A. Log attenuation of *Listeria monocytogenes* strains after irradiation (6 J/cm<sup>2</sup> UVA) at indicated S-59 concentration.

<i>Listeria</i> strain	0 nM S-59 Log titer	Log attenuation of <i>Listeria monocytogenes</i>			
		S-59 concentration (nM)			
		2	20	100	500
DP-L4017	7.81	0.66	2.58	>6.81	>6.81
L4017/ <i>uvrAB</i> clone 1	7.67	1.82	>6.67	>6.67	>6.67
L4017/ <i>uvrAB</i> clone 2	7.68	1.96	>6.68	>6.68	>6.68
DP-L4056	8.36	1.18	3.10	>6.36	>6.36
L4056/ <i>uvrAB</i> clone 1	7.65	2.04	>6.65	>6.65	>6.65

L4056/ <i>uvrAB</i> clone 2	7.84	1.96	>6.84	>6.84	>6.84
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Table 11B. Log attenuation of *Listeria monocytogenes* strains after irradiation (6 J/cm<sup>2</sup> UV A) at indicated S-59 concentration.

<i>Listeria</i> strain	Log attenuation of <i>Listeria monocytogenes</i>				
	0 nM S-59	S-59 concentration (nM)			
	Log titer	2	10	20	100
DP-L4017	8.62	0.56	0.97	2.33	>7.62
L4017/ <i>uvrAB</i> clone 1	8.67	1.09	4.44	>7.67	>7.67
DP-L4029	8.68	0.48	1.10	2.98	>7.68
L4029/ <i>uvrAB</i> clone 1	8.59	1.78	5.99	>7.59	>7.59
L4029/ <i>uvrAB</i> clone 2	8.63	1.50	6.60	>7.63	>7.63

[0180] The *uvrAB* mutant strains can be used directly as a parent strain in which to incorporate expression cassettes encoding heterologous antigens relevant to malignant or to infectious disease. In this configuration, following photochemical attenuation with S-59 and UVA light, the bacterium retains its ability to program MHC class I-restricted responses, because while the ability to replicate its DNA has been abrogated via cross-linking, the ability to express its genetic complement remains essentially intact. Furthermore, as a result of the requirement of significantly fewer DNA cross-links to inactivate *uvrAB* mutants, in the context of the population of bacterial genomes comprising a vaccine dose, the expression of any one gene will not be significantly affected, due to the low level of DNA crosslinking resulting in essentially no interruption of expression, at that given gene. Finally, the *uvrAB* mutation can be combined with any other attenuating mutation(s), in order to derive a safe and efficacious vaccine platform combining both photochemical and genetic attenuation. In the composition described herein, the *uvrA*, *uvrB*, or *uvrC* genes, or any *Listeria* gene involved in NER, alone, or in any combination, can be mutated such that a functional form of the protein is not expressed. These compositions can be used as an approach for deriving a safe and efficacious vaccine derived from a selected bacterial pathogen, in order to protect against challenge with the wild-type pathogen in vaccinated individuals. Alternatively, these compositions can be used as an approach for deriving a safe and efficacious recombinant vaccine platform for

the expression of heterologous antigens relevant to any selected infectious or malignant disease.

### EXAMPLE 8

Insertion of antigen expression cassettes into the genomes of selected *Listeria* strains by allelic exchange or by a site-specific integration vector

[0181] The strains described in Example 7, any selected *Listeria* strain, or any bacterial strain, can be modified further to express a heterologous protein or antigen relevant to malignant or infectious disease. Expression of the heterologous protein can be via a plasmid containing a replicon that is compatible with the selected host bacterium so that the plasmid is stably maintained. Alternatively, a prokaryotic expression cassette can be integrated stably into the genome of the host bacterium using a variety of methods, including allelic exchange as described in Example 7, or with vectors that integrate randomly or site-specifically, that are derived from selected transposons or bacteriophage.

[0182] As an example, the derivation of recombinant *Listeria monocytogenes* derived from the *uvrAB* nucleotide excision repair (NER) mutant strains described in Example 7, by utilizing a site-specific integration vector known as pPL2, which is derived from the listeriphage PSA (Phage from ScottA), is described here [Lauer et al., J. Bacteriol. 184:4177-4186 (2002)]. Specifically, the pPL2 integration vector is engineered to express the chicken ovalbumin (OVA) model antigen as a fusion partner with the amino-terminal half of the Listeriolysin O (LLO) protein that includes the secretion signal and PEST sequence [Decatur and Portnoy, Science 290:992-995 (2000)], but lacking hemolysin activity, that is fused in-frame with OVA. Expression of the truncated LLO-OVA fusion protein is driven by the *hly* promoter, which is a *prfA*-dependent promoter that drives the expression of *Listeria* virulence genes, including LLO. This vector is known as pPL2/LLO<sub>ss-PEST</sub>-OVA. The pPL2 vector integrates within the tRNA<sup>Arg</sup> gene of *Listeria* in such a manner that the native sequence of the tRNA gene is restored upon successful integration, thus keeping its native expressed function intact.

[0183] The first step in the construction of pPL2/LLO<sub>ss-PEST</sub>-OVA is to amplify the *hly* promoter and LLO<sub>ss-PEST</sub> sequence together from DP-L4056 wild-type *Listeria* genomic DNA, by PCR using the primer pair of forward primer *KpnI*-LLO nts. 1257-1276 (SEQ ID NO:12) and reverse primer *XhoI*-LLO1665R (SEQ ID NO:13). The 426 bp amplicon is purified with GeneClean, digested with *KpnI* and *XhoI*, and ligated into pPL2 plasmid,

which is prepared by digesting with KpnI and XhoI, treatment with calf intestinal alkaline phosphatase (CIAP), and purified with GeneClean. Correct plasmids containing the LLO<sub>ss</sub>-PEST sequence are verified by digesting with KpnI and XhoI and 1% agarose/TAE electrophoresis, yielding DNA fragments of 418 bps and 6112 bps. This intermediate plasmid DNA construct is known as pPL2/LLO<sub>ss</sub>-PEST.

[0184] The OVA sequence can be amplified by PCR from any number of plasmids that are used by those in the art, including pDP3616 plasmid DNA from DP-E3616 *E. coli* [Higgins et. al., Mol. Molbiol. 31:1631-1641 (1999)], using the primer pair of forward primer *XhoI-NcoI* OVA cDNA nts. 174-186 (SEQ ID NO:14) and reverse primer *XhoI-NotI-HindIII* (SEQ ID NO:15).

[0185] The 1013 bp amplicon is purified with GeneClean, digested with XhoI and NotI, and ligated into pPL2/LLO<sub>ss</sub>-PEST plasmid, prepared by digesting with XhoI and NotI, treatment with CIAP, and purified with GeneClean. The correct plasmid construct containing the LLO<sub>ss</sub>-PEST and OVA sequence is verified by digesting with KpnI, XhoI, and NotI and 1% agarose/TAE electrophoresis, yielding DNA fragments of 994 bps, 1560 bps, and 6039 bps. The precise expected sequence of the LLO and OVA regions of plasmid pPL2/LLO<sub>ss</sub>-PEST-OVA is confirmed by sequencing.

[0186] The pPL2/LLO<sub>ss</sub>-PEST-OVA plasmid is incorporated into the tRNA<sup>Arg</sup> gene in the genome of selected *Listeria uvrAB* mutant strains described in Example 7, exactly according to the methods as described previously [Lauer et. al., J. Bacteriol. 184, 4177-4186 (2002)]. Briefly, the plasmid pPL2/LLO<sub>ss</sub>-PEST-OVA is first introduced into the *E. coli* host strain SM10 (Simon et. al., Bio/Technology 1:784-791 (1983)) by electroporation or by chemical means. Subsequently, the pPL2/LLO<sub>ss</sub>-PEST-OVA plasmid is transferred from transformed SM10 to the selected *Listeria* strains by conjugation. Following incubation on drug-selective BHI agar plates containing 7.5 µg of chloramphenicol per ml and 200 µg of streptomycin per ml as described, selected colonies are purified by passaging 3 times on plates with the same composition. To verify integration of the pPL2 vector at the phage attachment site, individual colonies are picked and screened by PCR using the primer pair of forward primer NC16 (SEQ ID NO:16) and reverse primer PL95 (SEQ ID NO:17). Selected colonies having the pPL2/LLO<sub>ss</sub>-PEST-OVA plasmid incorporated into the tRNA<sup>Arg</sup> gene in the genome of selected *Listeria uvrAB* mutant strains will yield a diagnostic DNA amplicon of 499 bps.

[0187] The ability of the recombinant *Listeria uvrAB* mutants harboring a stable integrant of pPL2/LLO<sub>ss-PEST</sub>-OVA to be taken up by antigen presenting cells and subsequently program presentation of OVA via the MHC class I pathway is tested, using the cloned C57BL/6-derived dendritic cell line DC2.4, as described in Example 1. Presentation of OVA peptide by DC2.4 cells on class I molecules following phagocytosis of *Listeria* is measured after incubation with B3Z cells, also as described in Example 1. These procedures verify that the recombinant *Listeria* strains are functional, and can be used further as described in the Examples contained herein.

[0188] Thus, this example provides instructions for introducing a prokaryotic expression cassette encoding any desirable antigen(s) related to selected infectious and malignant diseases into DNA repair mutant *Listeria* strains containing a deletion within the *uvrAB* gene. The said recombinant *Listeria* strains can be inactivated by treatment with psoralens as described in Example 1 and can be used subsequently for a variety of applications, including, for example, prophylactic and therapeutic vaccines for infectious and malignant disease.

#### EXAMPLE 9

##### Bacterial Vaccines derived from nucleotide-excision repair (NER) mutants

[0189] The examples described in this patent illustrate the efficacy of vaccine compositions utilizing genomic inactivation through photochemical treatment of the recombinant delivery platform encoding antigens related to infectious and malignant disease. According to this composition, while the genomes are inactivated and cannot separate during replication, the transcriptional profile remains largely intact, thus resulting in antigen expression *de novo* in the vaccinated individual, and optimal induction of pathogen-specific immune responses, including CD8+ cytotoxic T cells (CTL). Furthermore, as described in Example 7, by utilizing a vaccine platform in this composition in which the DNA nucleotide excision repair (NER) machinery has been inactivated by any number of means, including by engineered genetic deletion, the sensitivity to photochemical inactivation in these mutants is dramatically increased.

[0190] As a result of the requirement of significantly fewer DNA cross-links to inactivate the DNA repair mutants, in the context of the population of bacterial genomes comprising a vaccine dose, the expression of any one gene will not be significantly

affected, due to the low level of DNA crosslinking resulting in essentially no interruption of expression, at that given gene.

[0191] Thus, the overall utility of gene-based vaccines utilizing bacterial platforms derived from pathogens can be increased dramatically by combining photochemical inactivation with a vector defective in NER. While the inactivated vaccine cannot cause disease, it still retains its efficient ability to induce potent immunity, including T-cell mediated cellular immunity, specific for the vector-expressed heterologous antigens. Furthermore, the *uvrAB* mutation can be combined with any other attenuating mutation(s), in order to derive a safe and efficacious vaccine platform combining both photochemical and genetic attenuation.

[0192] Significantly, these compositions can be used as an approach for deriving a safe and efficacious vaccine derived from a selected bacterial pathogen, in order to protect against challenge with the wild-type pathogen in vaccinated individuals. According to this application, it is not feasible in many cases to derive a safe and efficacious vaccine that is derived from an attenuated viable form of the pathogen, as the possibility for reactivity and disease pathogenesis in particular individuals receiving the vaccine remain high. While subunit or inactivated vaccines related to a selected bacterial pathogen might be safe, on the other hand, these vaccines are often not efficacious because they do not efficiently elicit the breadth, depth, and durability of pathogen-specific immune responses that are required to protect the vaccinated individual against challenge with the wild-type form of the said pathogen. Thus, it is well known in the art that there is a clear need for vaccine compositions that combine safety with an efficient ability to elicit the type of immune responses in vaccinated individuals that are protective.

[0193] As such, mutants in the nucleotide-excision repair (NER) pathway of pathogenic microbes provide a composition that can be used for safe and efficacious vaccines that elicit protection against challenge in immunized individuals with amounts of the said microbe that are sufficient to cause disease in non-vaccinated individuals. NER is catalyzed by an ATP-dependent nuclease made of three subunits, known as the ABC excinuclease, and encoded by the genes *uvrA*, *uvrB*, and *uvrC*. Mutations in any one or more than one of the three *uvr* genes results in cells, including microbes of pathogenic organisms, extremely sensitive to photochemical inactivation utilizing psoralens and UVA light.

[0194] As an example, mutation of the *uvr* genes of *Bacillus anthracis* (*B. anthracis*), the etiological agent of Anthrax, is provided. The current acellular anthrax

vaccines that are licensed for human use are based on sterile culture supernatants of attenuated *B. anthracis* adsorbed on alum hydroxide (U.S. vaccine), or precipitated with alum phosphate (U.K. vaccine). It is well known that these vaccines are rather weak, requiring at least six immunizations for protection as well as annual boosters.

[0195] In the composition described herein, the *uvrA*, *uvrB*, or *uvrC* genes, or any *B. anthracis* gene involved in NER, alone, or in any combination, is mutated such that a functional form of the protein is not expressed.

[0196] As an example, mutation in the *uvrA*, *uvrB*, or *uvrC* genes, or any *B. anthracis* gene involved in NER, can be performed, for example, by allelic exchange, as described in Example 7. While the *uvr* genes of *B. anthracis* have not been identified through targeted deletion and characterization of the phenotypes of the resulting mutant strains, the *uvr* genes can be identified through a homology search with the genomes of related organisms in whose *uvr* genes are known. For example, the genome of *B. anthracis*, that is, the main chromosome and the two virulence plasmids can be compared with *Bacillus Subtilis* (*B. Subtilis*), a related bacterium from the same genera as *B. anthracis*. The genomic scaffold representing the main chromosome of the Florida *B. anthracis* isolate (Read et. al. 2002. *Science* 296, 2028-2033) has a GenBank accession number of AAAC010000001. *B. subtilis* has a GenBank accession number of NC\_000964. The *B. subtilis uvrA* gene encompasses nts. 3609064 to 3611997, and the *B. subtilis uvrB* gene encompasses nts. 3612005-3613990. A BLAST search was performed using the *B. subtilis uvrA* and *uvrB* coding sequences against the *B. anthracis* sequence. This analysis identified a region of 72% sequence identity in the genome of *B. anthracis* that corresponds to the *uvrA* and *uvrB* genes of this organism. The *B. anthracis uvrA* gene maps from 226021-228783, and bears 72% sequence homology to the *B. subtilis uvrA* gene (2082/2867 identical sequence homology alignment). The *B. anthracis uvrB* gene maps from 228864-230771, and bears 72% sequence homology to the *B. subtilis uvrB* gene (1401/1925 identical sequence homology alignment). Thus, the *B. anthracis uvrAB* genes include nts. 226021 to 230771 of the main chromosome of *B. anthracis*.

[0197] Deletion of the *B. anthracis uvrAB* genes, including nts. 226021 to 230771 of the main bacterial chromosome can be accomplished according to the methods described in Example 7 for the deletion of *uvrAB* genes in *L. monocytogenes*. Briefly, this region and approximately 1000 bps both upstream and downstream of the *B. anthracis* genome are amplified by PCR, and subsequently cloned into the pKSV7 allelic exchange plasmid vector. As an alternative, a *Bacillus* genera-specific or *B. anthracis*-specific temperature-



sensitive (*ts*) replicon may be substituted for the *Listeria ts* replicon present in the pKSV7 allelic exchange plasmid vector. Using convenient restriction endonuclease recognition sites mapping specifically within the *uvrAB* region, any part of the *uvrA*, *uvrB*, or all of the *uvrAB* genes sequence are deleted. Finally, the allelic exchange plasmid is introduced into *B. anthracis* and NER mutants are selected as described in Example 7. Any selected *B. anthracis* strain can be used as a parent strain for derivation of the NER-defective vaccine, including, for example, the following strains: Ames, Vollum, A1.a/10, A1.b/23, A2/29, A3.a/34, A3.b/57, A4/69, B/80,  $\Delta$ sterne, VN41 $\Delta$ 1, Dames, NNRI  $\Delta$ 1, and DNH1. Additionally, other attenuating mutations can be incorporated into the genome of the selected NER mutant *B. anthracis* strain, to enable vaccine compositions combining photochemical with genetic inactivation. Such *B. anthracis* vaccine compositions are able to induce immune responses against known correlates of anthrax immunity and protection, including lethal factor (LF), edema factor (EF), and protective antigen (PA). Additionally, as a result that the expression profile of the NER mutant vaccine composition remains intact, immune responses against other unknown correlates of anthrax immunity and protection, including those expressed from the two virulence plasmids pXO1 and pXO2 and the main chromosome are also induced.

[0198] The compositions described herein, using *B. anthracis* as an example utilizing NER mutants as a component of vaccine, can be used in either a prophylactic or a therapeutic immunization setting against all three types of anthrax according to the route of infection, including cutaneous, gastrointestinal and respiratory. Furthermore it can be appreciated that the approach for generating NER mutants of *B. anthracis* to derive a safe and efficacious vaccine can be adopted to derive safe and efficacious vaccines for any microbial pathogen that utilizes NER.

#### EXAMPLE 10

Use of vaccines of the invention for the *in vivo* treatment of human cancers.

[0199] As an example of the treatment or prevention of a human cancer, a vaccine comprising a microbial population in which the microbial nucleic acid is modified so that the proliferation of the microbial population is attenuated, wherein the microbial gene expression is substantially unaffected, is administered to an individual. The microbe can be prepared following the protocols of examples 7 and 8, wherein any desired prokaryotic

expression cassettes encoding human tumor antigen(s) are incorporated into the microbe, by utilizing, for example the pPL2 integration vector described in Example 8, or any modifications thereof, or by any methods that are common to those in the art. The resulting population may be formulated in crude, or preferably purified form. They may be prepared as a liquid suspension or may be freeze-dried and resuspended in a suitable carrier for administration. In addition, they may be formulated with additives such as preservatives (e.g. thimerosal, 2-phenoxy ethanol), stabilizers (e.g. lactose, monosodium glutamate), adjuvants (e.g. aluminum hydroxide, aluminum phosphate, cytokines), antibiotics (e.g. neomycin, streptomycin) or other substances. Formulations may be resuspended or diluted in a suitable diluent such as sterile water, saline, isotonic buffered saline (e.g. phosphate buffered to physiological pH), or other suitable diluent.

[0200] The vaccine may be administered by a variety of routes, including oral, nasal, intravenous, intradermal, intraperitoneal, intramuscular, intralymphatic and subcutaneous routes, as well as by any route that is relevant for any given malignant or infectious disease. An effective amount of the vaccine will be administered to an individual for treatment. For a therapeutic treatment, an effective amount is a dose that will result in the desired immune response, wherein the immune response either slows the growth of the targeted tumors, reduces the size of the tumors, or preferably eliminates the tumors completely. The administration of the vaccine may be repeated at appropriate intervals, and may be administered simultaneously at multiple distinct sites in the vaccinated individual. For the prophylactic treatment, an effective amount is a dose that will result in a protective immune response such that the likelihood of an individual to develop the cancer is significantly reduced. The vaccination regimen may be comprised of a single dose, or may be repeated at suitable intervals until a protective immune response is established.

[0201] The therapeutic treatment of an individual may be started on an individual who has been diagnosed with a cancer as an initial treatment, or may be used in combination with other treatments. For example, individuals who have had tumors surgically removed or who have been treated with radiation therapy or by chemotherapy may be treated with the vaccine in order to reduce or eliminate any residual tumors in the individual, or to reduce the risk of a recurrence of the cancer. The prophylactic treatment of an individual would be started on an individual who has an increased risk of contracting certain cancers, either due to environmental conditions or genetic predisposition.

## EXAMPLE 11

Antigen presentation of *Listeria* strain DP-L4029 with and without *uvrAB* mutation following S-59 psoralen UVA treatment.

[0202] The *Listeria* strain DP-L4029 *uvrAB* mutant clone 1 of Example 7 was modified to express the OVA antigen using the procedure of Example 8. This strain and DP-L4029 modified to express OVA were treated with the psoralen S-59 at various concentrations. The *Listeria* strains were grown overnight at 37 °C and a 2 mL aliquot was diluted into 100 mL of BHI and grown approximately 4 hours at 37 °C to an OD<sub>600</sub> of 0.5 (approximately  $1 \times 10^9$  CFU/mL). A 5 mL aliquot of each *Listeria* strain was added to a 15 mL tube and centrifuged for 20 minutes at 2300 x g, the supernatant removed, and the bacteria resuspended in 5 mL of PBS resulting in approximately  $1 \times 10^9$  CFU/mL. For the *uvrAB* mutant strain, 3 mM S-59 stock was diluted 33.3 µL to 10 mL PBS to give a 10 µM solution, and appropriate aliquots of this was added to the *Listeria* to final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 nM, while for the DP-L4029, S-59 was added to final concentrations of 100, 200, 400, 800, and 1000 nM in a final volume of 5 mL. These were transferred to a 6 well culture plate and irradiated for a dose of 0.5 J/cm<sup>2</sup> (FX101 9 UVA device). The samples were transferred to 15 mL tubes, 5 mL PBS was added, and they were centrifuged for 20 minutes at 2300 x g to wash out unreacted psoralen. The supernatant was removed and the bacteria resuspended in 5 mL PBS and transferred to new 6 well plates. These were irradiated at a UVA dose of 5.5 J/cm<sup>2</sup> in order to convert psoralen monoadducts to crosslinks. A sample of each *Listeria* strain was also heat killed by treating at 72 °C for 3 hours. The log titer and OVA antigen presentation were assessed as per Example 1. The results for the S-59 treated samples are found in Table 12A and Figures 9A and 9B (antigen presentation at 1 *Listeria* per DC 2.4 cell, calculated without subtracting background levels). The results for both heat killed strains showed a titer below the limit of detection (complete inactivation) and the heat killed bacteria did not present OVA antigen in the B3Z assay. The results indicate that the *uvrAB* mutant shows very strong antigen presentation even with attenuation of proliferation to the limit of detection where the non *uvrAB* mutant strain shows a greater reduction in the antigen presentation as a function of attenuation of proliferation (to approximately background levels with essentially complete inactivation). This demonstrates that the *uvrAB* mutant retains MHC class I presentation in the context of psoralen attenuated *Listeria* and should

provide a vaccine with an effective immune response and significantly increased level of safety.

Table 12A Log attenuation and OVA antigen presentation of *Listeria* strains UVA treated with varying concentrations of psoralen S-59.

[S-59] nM	Log attenuation		% OVA antigen presented*	
	DP-L4029- OVA	DP-L4029 <i>uvrAB</i> -OVA	DP-L4029- OVA	DP-L4029 <i>uvrAB</i> -OVA
10		2.47		84
20		3.93		84
30		5.28		76
40		6.44		76
50		6.92		68
60		>7.62		84
70		>7.62		84
80		>7.62		88
90		>7.62		92
100	3.85	>7.62	50	92
200	5.48		47	
400	6.78		19	
800	>7.78		13	
1000	>7.78		13	

\* As percent of untreated, measured at 1 *Listeria* per DC 2.4 cell.

[0203] Another study was done using the same strains. In this study the *Listeria* were grown in BHI at 37 °C overnight. These were diluted 1:50 into BHI and grown at 37 °C at 300 rpm to an OD<sub>600</sub> of 0.5, at which point 50 mL of solution was transferred to a clean flask and S-59 was added to the levels indicated in Table 12B. These samples were incubated at 37 °C at 300 rpm for approximately 1 hour (OD<sub>600</sub> approximately 1.0, approximately 1 x 10<sup>9</sup>/mL). A 1 mL aliquot was removed to assess the titer and the remaining was transferred to a 150 mm Petri dish and irradiated at a dose of 6 J/cm<sup>2</sup> (FX1019). The titer post irradiation was determined for each sample and the OVA antigen presentation was assessed as above. The results are found in Table 12B and Figures 9C and 9D (antigen presentation at 10 *Listeria* per DC 2.4 cell, calculated without subtracting background levels). The results indicate that for the parent strain, the antigen presentation

is at background levels where there is essentially complete inactivation whereas for the *uvrAB* mutant, there is an approximately 10-fold range of S-59 concentration over which there is essentially complete inactivation along with adequate antigen presentation.

Table 12B Log attenuation and OVA antigen presentation of *Listeria* strains UVA treated with varying concentrations of psoralen S-59 present during growth of the bacteria.

[S-59] μM	Log attenuation		% OVA antigen presented*	
	DP-L4029-OVA	DP-L4029 <i>uvrAB</i> -OVA	DP-L4029-OVA	DP-L4029 <i>uvrAB</i> -OVA
0.025		3.64		91
0.05		5.70		86
0.1		>8.10		87
0.2		>8.10		86
0.25	2.00		50	
0.4		>8.10		74
0.5	5.28		31	
0.8		>8.10		50
1.0	7.57		14	
1.6		>8.10		35
2.0	>8.38		11	
3.2		>8.10		16
4.0	>8.38		10	
6.4		>8.10		11
8.0	>8.38		10	
16.0	>8.38		11	

\* As percent of untreated, measured at 10 *Listeria* per DC 2.4 cell.

#### EXAMPLE 12

Protein synthesis in S-59/UVA treated *Listeria monocytogenes* DP-L4029 compared to DP-L4029 *uvrAB*.

[0204] *Listeria monocytogenes* DP-L4029 and DP-L4029 *uvrAB* were grown in BHI at 37 °C overnight. These were diluted 1:50 into BHI and grown at 37 °C at 300 rpm to an

OD<sub>600</sub> of 0.5, at which point 50 mL of solution was transferred to a clean flask and S-59 was added to a level of 2500 nM for the 4029 and 200 nM for the 4029 *uvrAB* mutant strain. These samples were incubated at 37 °C at 300 rpm for approximately 1 hour (OD<sub>600</sub> approximately 1.0). A 1 mL aliquot was removed to assess the titer and the remaining was transferred to a 150 mm Petri dish and irradiated at a dose of 6 J/cm<sup>2</sup> (FX1019). The titer post irradiation was determined for each sample to assess the level of inactivation, resulting in essentially complete inactivation. It has been determined that this treatment is approximately the lowest S-59 dose that provides inactivation to the limit of detection for both strains. For each strain, 1 x 10<sup>10</sup> bacteria, based on the OD<sub>600</sub> vs. titer CFU/mL growth curve, was transferred to a 15 mL centrifuge tube. The sample was centrifuged at 4 °C for 20 minutes at 2300 x g, the supernatant removed and the pellet washed with 50 mL of PBS. This was repeated for a total of three washes. The final pellet was suspended in 2 mL of DMEM without methionine or cysteine (Gibco) and incubated at 37 °C in 5% CO<sub>2</sub> incubator with shaking for 30 minutes. The samples were centrifuged in 2 mL centrifuge tubes at 1600 rpm for 2 minutes, the supernatant removed and 2 mL of DMEM without methionine or cysteine was added. An 80 µCi aliquot of <sup>35</sup>S methionine-cysteine was added (Perkin Elmer Life Sciences) and the sample incubated at 37 °C in 5% CO<sub>2</sub> incubator with shaking for 30 minutes. The samples were centrifuged as above and the supernatant removed. A 50 µL aliquot of each supernatant were loaded in adjacent lanes onto an SDS-PAGE gel (Invitrogen, NuPage 4-12% Bis-Tris gel) and run at 100 volts for approximately 1.5 hours. The gel was fixed with 10% acetic acid and 30% ethanol, then soaked in enhancer (Enlightning, NEN Life Sciences) for 15 minutes. The gel was dried for 3 hours at 80 °C and the bands visualized by exposure to X-ray film. The results for two studies are shown in Figure 10, indicating considerable protein synthesis in the *uvrAB* mutant strain while the parent strain shows limited protein synthesis.

### EXAMPLE 13

Comparison of S-59/UVA inactivation with or without S-59 present during growth of *Listeria*.

[0205] Two inactivation methods were compared with respect to inactivation of *Listeria monocytogenes* strains. In the first method, the *Listeria* was grown in BHI at 37 °C at 300 rpm overnight, then diluted 1:50 into BHI and grown at 37 °C at 300 rpm to an

OD<sub>600</sub> of 0.7-1.00. These were centrifuged and suspended in PBS with 1% BSA to a level of  $1 \times 10^9$ /mL. S-59 was added to a level of 120 nM for the parent strain and 30 nM for the *uvrAB* mutant strain. The samples were incubated on ice for approximately 60 minutes, then transferred to a 150 mm Petri dish and irradiated at a dose of 6 J/cm<sup>2</sup> (FX1019). In the second method, the *Listeria* was similarly grown to an OD<sub>600</sub> of 0.5, at which point 50 mL of solution was transferred to a clean flask and S-59 was added to a level of 2500 nM for the parent strain and 200 nM for the *uvrAB* mutant strain. These samples were incubated at 37 °C at 300 rpm for approximately 1 hour (OD<sub>600</sub> approximately 1.0, approximately  $1 \times 10^9$ /mL). A 1 mL aliquot was removed to assess the titer and the remaining was transferred to a 150 mm Petri dish and irradiated as per the first method. The titer post irradiation was determined for each sample, resulting in essentially complete inhibition of proliferation for all samples (>8 log inactivated). In a study done with DP-L4029 vs. DP-L4029*uvrAB*, the entire sample containing approximately  $1 \times 10^{11}$  bacteria treated by the second method, the entire sample was plated, indicating approximately 9 log kill for the parent strain and >10 log kill for the *uvrAB* mutant. The results on four different preparations of *Listeria* are given in Table 13.

Table 13 Inactivation of *Listeria monocytogenes actA* and *actA-uvrAB* with S-59/UV A, measurement of entire sample to assess log titer inactivation.

	Batch	Titer treated	Residual colonies	Log inactivation
<i>actA</i> - 2.5 μM S-59 6 J/cm <sup>2</sup>	1	$1.0 \times 10^{11}$	100	9
	2	$1.1 \times 10^{11}$	28	9.6
	3	$1.1 \times 10^{11}$	200	8.7
	4	$1.1 \times 10^{11}$	160	8.8
<i>actA-uvrAB</i> 200 nM S-59 6 J/cm <sup>2</sup>	1	$1.0 \times 10^{11}$	0	11
	2	$1.1 \times 10^{11}$	11	10
	3	$1.1 \times 10^{11}$	0	11
	4	$1.1 \times 10^{11}$	1	11

[0206] In one study, the two methods were compared using *Listeria monocytogenes* DP-L4029-OVA and DP-L4029 *uvrAB*-OVA. Samples were prepared as above and were centrifuged for 20 minutes at 2300 x g, the supernatant removed and the bacteria washed once with PBS. After centrifuging and removing the PBS wash, the final pellet was

resuspended in 8 % DMSO in PBS, then quickly frozen in cryo-vials, either using liquid nitrogen or dry ice, and stored at -80 °C. Sets of three mice (C57Bl/6) were injected intravenously with  $1 \times 10^8$  *Listeria* in 200  $\mu$ L (frozen stock diluted approximately 1:40 into HBSS). In addition to the S-59/UVA treated strains, injections were made with live and heat killed DP-L4029 *uvrAB*-OVA, as well as HBSS control. For the comparison of the two S-59 methods, mice were injected at day 0. For the samples prepared by the second method, additional sets of mice were injected again either on days 2 and 3 or on days 2, 3, 4, and 5. All mice were sacrificed on day 7 post vaccination and the spleens removed for analysis. The spleen cells were assessed for an OVA specific immune response by ELISPOT assay as described in Example 5, stimulating the cell population with SL8 (OVA specific). The results are shown in figure 11A, indicating that the *Listeria* prepared by the second method, for both the parental strain and the *uvrAB* mutant, result in a more potent OVA specific immune response than for strains prepared by the first method. The ELISPOT assay was also done with stimulation using LLO class II antigen LLO190, or class I antigen LLO296. The ELISPOT results comparing all three antigens is shown in Figure 11B, indicating that the LLO specific CD 4<sup>+</sup> response is similar to the OVA specific response. The spleen cells were also assessed by ICS as described in Example 5, stimulating with either SL8, LLO190, or LLO296. The results are shown in Figures 12A-C, indicating a stronger immune response for both OVA and LLO in the second method. The data also demonstrates the improved response for the *uvrAB* strain over the parent strain. In both strains, additional vaccination on successive days results in improved response to both OVA and LLO antigens (1 vs. 3 days).

[0207] In another study, DP-L4029 and DP-L4029 *uvrAB* strains are assessed for their ability to provide protective immunity against a wild type challenge in mice. Balb/c mice were vaccinated in groups as described in Table 14 with HBSS, DP-L4056 wild type (+/- heat killed), DP-L4027 (LLO deletion), DP-L4029 S-59/UVA treated (first and second methods as above), DP-L4029*uvrAB* S-59/UVA treated (first and second methods as above). Twenty-seven days after the vaccination, three mice per group were challenged with 2xLD<sub>50</sub> and six mice per group with 100xLD<sub>50</sub> of wild type *Listeria monocytogenes*. Three days post challenge, the mice challenged with 2xLD<sub>50</sub> were sacrificed and the spleen and liver were isolated and cultured for growth of *Listeria*. The spleen or liver from each mouse was homogenized in sterile distilled water with 0.5% Triton X-100 (Sigma). Serial 10-fold dilutions were plated on BHI agar plates containing streptomycin (50  $\mu$ g/mL) and



incubated at 37 °C overnight. The number of colony forming units per spleen or liver was determined as an indication of immunity to the wild type challenge. Figures 13A,B show that S-59/UVA treated samples give approximately 3 log reduction in CFU per organ compared to HBSS (non-vaccinated) controls, with the samples prepared by the second method showing more reduction in CFU than those prepared with the first method. In addition, the treated *uvrAB* mutant strain shows slightly better CFU reduction than the treated parent strain. While the CFU reduction is not as good as vaccination with wild type, the S-59/UVA treated strains show some efficacy for reduction in CFU, which generally correlates with protective immunity. The six mice challenged with 100xLD<sub>50</sub> were monitored for survival for ten days, with only the mice vaccinated with wild type *Listeria* surviving.

Table 14 Dosing of Balb/c mice for assessment of protective immunity comparing two S-59/UVA methods.

Vaccine composition	S-59 / 6 J/cm <sup>2</sup> UVA Method	Dose of vaccination <sup>a</sup> (200 µL IV)
HBSS	-	-
DP-L4056	-	5 x 10 <sup>3</sup>
DP-L4027	-	1 x 10 <sup>8</sup>
DP-L4029	Method 1 (120 nM S-59 in PBS)	1 x 10 <sup>8</sup>
DP-L4029	Method 2 (2500 nM S-59 in BHI)	1 x 10 <sup>8</sup>
DP-L4029 <i>uvrAB</i>	Method 1 (30 nM S-59 in PBS)	1 x 10 <sup>8</sup>
DP-L4029 <i>uvrAB</i>	Method 2 (200 nM S-59 in BHI)	1 x 10 <sup>8</sup>
DP-L4056 heat killed	-	1 x 10 <sup>9</sup>

[0208] An additional study was done in Balb/c mice using HBSS, DP-L4056 wild type (+/- heat killed), DP-L4027 (LLO deletion), DP-L4406*actA* (*actA/inlB* deletion double mutant, deposited on October 3, 2003, ATCC number PTA-5562) DP-L4029 +S-59/UVA (second method), DP-L4029*uvrAB* +/- S-59/UVA treated (second method only) or + heat killed, where vaccination was done daily for 1, 3, or 5 days for S-59 and heat killed strains. The dosing is summarized in Table 15. Twenty-nine days post the first vaccination, three mice from each group were challenged with 20x LD<sub>50</sub>, and six from each group were challenged with 100x LD<sub>50</sub> of wild type *Listeria monocytogenes*. These mice were monitored for survival for ten days. Thirty-two days post first vaccination, three additional

mice from each group were challenged with  $2 \times \text{LD}_{50}$  of wild type and three days later sacrificed and the spleen and liver were isolated and cultured for growth of *Listeria*. In addition, the anti-*Listeria* antibody titer of the mice sera was assessed by doing an ELISA assay. Frozen, ground *Listeria* in a sodium bicarbonate buffer was plated and incubated with serum from the vaccinated mice with serial dilutions, then bound antibody was detected with goat anti-mouse antibody conjugated to HRP. An HRP substrate was added and the level of antibody determined by quantitatively measuring the color change. These were compared to naïve mice to assess *Listeria* specific antibody, where a sample was considered positive for *Listeria* if greater than one standard deviation above the measurement of a naïve serum sample. The CFU per spleen or liver results are shown in Figures 14A,B, the anti-*Listeria* antibody titer is shown in Figure 15, and the survival results are shown in Figure 16. This study demonstrates good CFU reduction and protective immunity of the S-59 treated *uvrAB* strain with 3 or 5 vaccinations, approaching that of the untreated *uvrAB* strain, and is nearly as effective as the wild type strain.

Table 15 Dosing of Balb/c mice for assessment of protective immunity, multiple vaccinations with S-59/UVA treated strains.

Vaccine composition	Treatment	Days vaccinated	Dose of vaccination (200 $\mu\text{L}$ IV)
HBSS	-	1	-
DP-L4056	-	1	$5 \times 10^3$
DP-L4056 heat killed	-	1	$1 \times 10^9$
DP-L4029	S-59 Method 2	1	$1 \times 10^8$
DP-L4029 <i>uvrAB</i>	S-59 Method 2	1	$1 \times 10^8$
DP-L4029 <i>uvrAB</i>	S-59 Method 2	3	$1 \times 10^8$ (day0) $2 \times 10^7$ (day 2-3)
DP-L4029 <i>uvrAB</i>	S-59 Method 2	5	$1 \times 10^8$ (day0) $4 \times 10^7$ (day 2-5)
DP-L4029 <i>uvrAB</i>	-	1	$5 \times 10^6$
DP-L4029 <i>uvrAB</i>	Heat killed	1	$1 \times 10^9$
DP-L4029 <i>uvrAB</i>	Heat killed	3	$1 \times 10^9$ (day0) $2 \times 10^8$ (day 2-3)
DP-L4029 <i>uvrAB</i>	Heat killed	5	$1 \times 10^9$ (day0) $4 \times 10^8$ (day 2-5)

DP-L4027	-	1	$1 \times 10^8$
DP-L4406 <i>actA</i>		1	$5 \times 10^7$

## EXAMPLE 14

Demonstration of breaking of immune tolerance using S-59/UVA treated strains in a mouse model.

[0209] DP-L4029 and DP-L4029 *uvrAB* strains expressing Gp-70-AH1A5 and OVA were S-59/UVA treated according to the second method of Example 13. Gp-70 is an autologous mouse antigen that is expressed by CT-26 tumor cells. The AH1A5 is a single base mutation of the natural sequence which has been shown to induce an immune response when expressed in live strains (AH1 peptide is SPSYVYHQF (SEQ ID NO:20), AH1A5 peptide is SPSYAYHQF (SEQ ID NO:21)). In a prophylactic immunization study, Balb/c mice were vaccinated intravenously (100  $\mu$ L) in groups of 8 mice according to Table 16 (day 7 post the first set of vaccinations, 3 mice per group were sacrificed and the spleens harvested). At day 21 post initial vaccination, the remaining 5 mice per group were injected intravenously with  $1 \times 10^5$  CT-26 colon epithelial tumor cells (ATCC) and monitored for survival.

Table 16 Vaccine strains and treatment regimen.

Group	Vaccine strain	Treatment	Dosing day	Dose per injection
1	HBSS control	-	0, 14, 15	-
2	DP-L4029	-	0, 14, 15	$1 \times 10^7$
3	DP-L4029 AH1A5/OVA	-	0, 14, 15	$1 \times 10^7$
4	DP-L4029 AH1A5/OVA	Heat killed	0, 14	$3 \times 10^8$
5	DP-L4029 AH1A5/OVA	Heat killed	0, 1, 2, 14	$1 \times 10^8$
6	DP-L4029 AH1A5/OVA	S-59/UVA	0, 14	$3 \times 10^7$
7	DP-L4029 AH1A5/OVA	S-59/UVA	0, 1, 2, 14	$1 \times 10^7$
8	DP-L4029 <i>uvrAB</i> AH1A5/OVA	-	0, 14, 15	$1 \times 10^7$
9	DP-L4029 <i>uvrAB</i> AH1A5/OVA	Heat killed	0, 14	$3 \times 10^8$
10	DP-L4029 <i>uvrAB</i> AH1A5/OVA	Heat killed	0, 1, 2, 14	$1 \times 10^8$
11	DP-L4029 <i>uvrAB</i> AH1A5/OVA	S-59/UVA	0, 14	$3 \times 10^7$
12	DP-L4029 <i>uvrAB</i> AH1A5/OVA	S-59/UVA	0, 1, 2, 14	$1 \times 10^7$

[0210] The T cell population of the harvested spleen cells was assessed by ICS according to Example 5, using LLO91, AH1, AH1/A5 peptides or P815 and CT26 cells (completely inactivated with 150 mM S-59 and 3 J/cm<sup>2</sup> UVA) to stimulate the cells. The P815 cells serve as a negative control for CT26 whole cell stimulation, as the P815 does not express gp70 antigen. The results are shown in Figure 17, indicating that the treated *uvrAB* mutants result in an AH1A5 or AH1 specific response that can be improved with additional vaccinations. The cells were also assessed by ELISPOT assay according to Example 5. The cells were stimulated with either AH1A5 or AH1 peptides. The results are shown in Figure 18A, B indicating an immune response to both the AH1A5 and the AH1 with the *uvrAB* mutant strains.

#### EXAMPLE 15

Therapeutic vaccination of mice using psoralen attenuated *Listeria* strains with *uvrAB* deletion.

[0211] Using C57Bl/6 mice, B16.F10.M05.10.H3 (OVA+, this is a subclone of the cells used in Example 4 which have increased homogeneity for OVA expression) melanoma tumor cells were injected into the mice ( $1 \times 10^6$  in 100  $\mu$ L HBSS IV) to establish lung metastases. *Listeria monocytogenes* strains DP-L4029-OVA, DP-L4027-OVA, DP-L4038-OVA (*actA*/461T double mutant), and DP-L4029*uvrAB*-OVA were used for vaccinating groups of ten mice. The DP-L4029*uvrAB*-OVA strain was used with and without S-59 treatment (>8 log kill by first method of Example 13) and heat killed DP-L4029-OVA was used as a control along with HBSS only. The mice were vaccinated (100  $\mu$ L IV in HBSS) on day 3 post tumor implant with the dose given in Table 16. Thirty days post tumor implant, five mice per group were sacrificed and the lungs harvested. The number of metastases per lung were counted. The remaining five mice per group were monitored for survival. The number of lung metastases and median survival days are indicated in Table 17. The lungs for the *actA*<sup>+</sup>, *actA*<sup>-</sup> OVA, and *actA*<sup>-</sup>*uvrAB* OVA S-59/UVA treated and heat killed are shown in Figure 19A, number of lung metastases plotted in Figure 19B, and the survival is plotted in Figure 19C. This data shows that the S-59/UVA treated *uvrAB* mutant can be administered as a therapeutic vaccine, resulting in

significantly reduced lung metastases and extended survival compared to non-vaccinated, heat killed control, or DP-L4029 without OVA.

Table 17 Therapeutic vaccination of mice in an OVA lung tumor model.

Vaccine strain	Dose (CFU)	Mean # of lung mets per lung	Median survival days
HBSS	-	173	34
DP-L4029	$2 \times 10^7$	81	39
DP-L4029-OVA	$2 \times 10^7$	3	51
DP-L4029-OVA heat killed	$1 \times 10^9$	250	32
DP-L4029 $uvrAB$ -OVA	$2 \times 10^7$	3	53
DP-L4029 $uvrAB$ -OVA	$2 \times 10^5$	4	45
DP-L4029 $uvrAB$ -OVA (S-59)	$1 \times 10^9$	11	45
DP-L4029 $uvrAB$ -OVA (S-59)	$2 \times 10^5$	134	36
DP-L4027-OVA	$2 \times 10^7$	2	48
DP-L4038-OVA	$2 \times 10^7$	52	51

#### EXAMPLE 16

Therapeutic vaccination with S-59 inactivated *Listeria* strains expressing gp70 mouse antigen.

[0212] Using Balb/c mice, CT26 tumor cells (which express AH1) modified to express a human antigen (the human antigen being irrelevant for this experiment) were injected into the mice ( $2 \times 10^5$  in 100  $\mu$ L IV in HBSS) to establish lung metastases. *Listeria monocytogenes* strains DP-L4029, DP-L4029-AH1A5, DP-L4029 $uvrAB$ -AH1A5, and DP-L4406 $actA$ -AH1A5( $actA/inlB$  double mutant) were used for vaccinating groups of thirteen mice. The AH1A5 strains also express the OVA antigen. The DP-L4029 $uvrAB$ -AH1A5 strain was used without treatment, heat killed, or S-59 treated (per second method of Example 13). The mice were vaccinated (100  $\mu$ L HBSS IV) beginning 4 days after the tumor implant according to Table 18. Nineteen days post tumor implant, three mice per group were sacrificed and the lungs harvested. The number of metastases per lung were counted. The remaining ten mice per group were monitored for survival. The results for the lung metastases are shown in Figure 20A (lung pictures) and 20B (number of lung

metastases plotted) and survival is indicated in Table 18 and Figure 20C (*ΔactA* samples) and 20D (*ΔactAΔuvrAB* samples). The AH1A5 antigen is endogenous to the mice, such that any immunization effect would be breaking immune tolerance in the mice. The results indicate that the S-59 treated *uvrAB* mutant strain is able to break tolerance in the mice, resulting in significantly reduced lung metastases and extended survival. The therapeutic effect is improved when the vaccine is dosed over three days compared to a single vaccination (total dose delivered over three days is equal to the single day).

Table 18 Therapeutic vaccination of mice using *Listeria* modified to express AH1A5.

Vaccination strain	Vaccination days	Dose (CFU)	Med. Survival (days)	# survivors day 43
HBSS	Day 4	-	22	0
DP-L4029	Day 4	$1 \times 10^7$	24	0
DP-L4029-AH1A5	Day 4	$1 \times 10^7$	>43	10
DP-L4029-AH1A5 heat killed	Day 4	$3 \times 10^8$	21	0
DP-L4029-AH1A5 heat killed	Day 4, 5, 6	$1 \times 10^8$	22.5	1
DP-L4029-AH1A5 S-59/UVA	Day 4	$3 \times 10^7$	27.5	3
DP-L4029-AH1A5 S-59/UVA	Day 4, 5, 6	$1 \times 10^7$	23.5	2
DP-L4029 <i>uvrAB</i> -AH1A5	Day 4	$1 \times 10^7$	>43	10
DP-L4029 <i>uvrAB</i> -AH1A5 heat killed	Day 4	$3 \times 10^8$	23	1
DP-L4029 <i>uvrAB</i> -AH1A5 heat killed	Day 4, 5, 6	$1 \times 10^8$	24	1
DP-L4029 <i>uvrAB</i> -AH1A5 S-59/UVA	Day 4	$3 \times 10^7$	31	4
DP-L4029 <i>uvrAB</i> -AH1A5 S-59/UVA	Day 4, 5, 6	$1 \times 10^7$	>43	8
DP-L4406 <i>actA</i> -AH1A5	Day 4	$1 \times 10^7$	>43	10

## EXAMPLE 17

Evaluation of S-59/UVA treated *Listeria monocytogenes* localization in dendritic cells using fluorescence microscopy.

[0213] The uptake and distribution of *Listeria monocytogenes* within an antigen presenting cell was evaluated by fluorescence microscopy. The dendritic cell line DC 2.4 was cultured on coverslips in a Petri dish at  $5 \times 10^5$  cells per dish in complete RPMI media, RPMI-1640 (Gibco) supplemented with 10% FBS (Hyclone), 1x Non-Essential Amino Acids (Cellgro),  $5 \times 10^4$  I.U. Penicillin/ $5 \times 10^4$   $\mu$ g Streptomycin (Irvine Scientific), 2 mM L-glutamine (Irvine Scientific) and 1 mM Sodium Pyruvate (Sigma), and incubated overnight at 37 °C (this could be done similarly with other cell lines, e.g. macrophage J774). Stationary phase cultures of *Listeria* strains (DP-L4056, DP-L4027 (LLO-) and DP-L4056uvrAB) were prepared by seeding 3 mL of BHI media with a bacterial colony and growing at 30 °C overnight.

[0214] The overnight cultures of *Listeria* were diluted 1:20 in fresh BHI media and stationary phase cultures at 30°C were grown to an OD<sub>600</sub> of 0.5-0.6. Approximately 1 mL of the overnight cultures for the DP-L4056 and DP-L4056uvrAB strains were also heat killed at 72 °C for 3-4 hrs. Frozen stocks of psoralen inactivated DP-L4056 and DP-L4056uvrAB *Listeria*, prepared according to the second method of Example 13, were thawed and allowed to recover in stationary phase at 37° C for 1 hr. Prior to infection, OD<sub>600</sub> readings of all *Listeria* preparations were obtained, the number of DC 2.4 cells per coverslip were counted and Multiplicity of Infection (MOI, number of bacteria per DC 2.4 cell) for each strain were calculated. Fresh log phase cultures were used to infect cells at an MOI of 5, heat-killed cultures were used at an MOI of 20 and S-59/UVA treated strains at an MOI of 10.

[0215] The coverslips were transferred to a 24 well dish and washed 3 times with RPMI lacking Pen/Strep and appropriate dilutions of the *Listeria* strains to give the desired MOI were incubated with the cells in Pen/Strep free media for 30 mins at 37° C. The coverslips were then washed 3 times with Pen/Strep free media and incubated at 37° C for another 30 mins. At the end of the incubation, the coverslips were washed and incubated in media with 50 $\mu$ g/ml Gentamycin for 4 hrs at 37 °C. Coverslips were then washed in PBS and fixed in 3.5% formaldehyde/PBS for 15 mins at room temperature. Post fixation,

coverslips were washed/permeablized with TBS-Tx buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100) and blocked in 1 % BSA/TBS-Tx for 15 mins at room temperature. Coverslips were stained with Rabbit anti-*Listeria* O antigen anti-serum (Difco) for 30 minutes at room temperature and washed in TBS-Tx buffer. Samples were then stained with Fluorescein labeled anti-rabbit secondary antibody (Vector Laboratories) and actin was stained with Rhodamine-Phalloidin (Molecular Probes). Coverslips were washed in TBS-Tx and mounted on slides in VectaShield+DAPI-hardset (Vector Laboratories) in order to stain for cell nuclei. Slides were allowed to dry for at least 8 hrs and cells were visualized on a Nikon TE300-U inverted microscope. Images were taken using a CCD Hamamatsu C4742-95-12NR camera and analyzed using Image-Pro software from Phase 3 Imaging Systems.

[0216] Three images were taken for each field; one using a UV-2E/C filter (CHROMA Technology Corp, visualizes DAPI/nuclei); a second with a HYQ TRITC filter (CHROMA Technology Corp, visualizing actin) and a third using a B-1A (HYQ-FITC) filter (CHROMA Technology Corp, visualizing *Listeria*). The three images were then merged to determine if staining for *Listeria* co-localizes with staining for actin. *Listeria* that were unable to escape the phagolysosome appear green while those that were able to escape into the cytosol were able to nucleate actin and therefore appeared yellow due to the co-localization of actin (red) and *Listeria* (green). In order to quantitate the percentage of *Listeria* that was able to escape the lysosome, the total number of *Listeria* in the field were counted and the number of *Listeria* that appeared yellow were determined by counting yellow bacteria or by confirming the presence of the actin from the rhodamine image (see Figure 21A). The number of *Listeria* that escaped the phagolysosome were divided by the total number of *Listeria* counted and the percentage of phagolysosomal escape was calculated, as reported in Table 19 and represented in Figure 21B. The results indicate that the heat killed strains and the S-59/UVA treated wild type strain behave like the LLO strain, i.e. can not escape the phagolysosome, while the *uvrAB* mutant that is S-59/UVA treated shows substantial ability to escape the phagolysosome.

Table 19 Percentage of *Listeria* escaping the phagolysosome for DP-L4056 (+/- S-59/UVA, heat killed), DP-L4027, and DP-L4056uvrAB (+/- S-59/UVA, heat killed).

<i>Listeria</i> Strain	Treatment	<i>Listeria</i> counted	Cytoplasmic <i>Listeria</i>	% Phagolysosomal escape



DP-L4056	none	855	521	61
DP-L4056	Heat killed	189	0	0
DP-L4056	S-59/UVA	642	1	0.16
DP-L4056 <i>uvrAB</i>	none	795	470	59
DP-L4056 <i>uvrAB</i>	Heat killed	162	0	0
DP-L4056 <i>uvrAB</i>	S-59/UVA	1047	493	46.9
DP-L4027	none	343	5	1.4

## EXAMPLE 18

Visualization of S-59 UVA treated *Listeria monocytogenes uvrAB* strains using Gram stain.

[0217] Wild-type and *uvrAB* strains of *Listeria monocytogenes* were grown to an OD<sub>600</sub> of 0.5, at which point 50 mL of solution was transferred to a clean flask and S-59 was added to a level of 2500 nM for the wild-type strain and 200 nM for the *uvrAB* mutant strain. These samples were incubated at 37 °C at 300 rpm for approximately 1 hour (OD<sub>600</sub> approximately 1.0, approximately 1 x 10<sup>9</sup>/mL). A 1 mL aliquot was removed to assess the titer and the remaining was transferred to a 150 mm Petri dish and irradiated at a UVA dose of 6 J/cm<sup>2</sup> (FX-1019), resulting in > 8 log inactivation for both strains. The treated strains were stored frozen as described in Example 13. These were thawed and diluted 1:10 into BHI medium in a 15 mL tube at a concentration of approximately 1-2 x 10<sup>9</sup> per mL. These were incubated at 37 °C at 300 rpm and aliquots were removed at 0, 2, 4, 6, 8 hours and overnight (approximately 18 hours). The aliquots were spread on glass slides (approximately 50 µL) and allowed to air dry. The smear was heat fixed by passing through a flame three times, then allowed to cool before Gram staining using Fisher Gram Stain Set (catalog # 282-407). The slides were viewed on a microscope and photographed and the negative images are shown in Figure 22. This clearly demonstrates the unique nature of the treated repair deficient strain, which shows chains indicating gene expression but is not able to divide such that the bacteria do not proliferate.

## EXAMPLE 19

Construction of additional mutant *Listeria* strains.

[0218] *Preparation of mutant Listeria strains.* *Listeria* strains were derived from 10403S (Bishop et al., *J. Immunol.* 139:2005 (1987)). *Listeria* strains with in-frame deletions of the indicated genes were generated by SOE-PCR and allelic exchange with established methods (Camilli, et al, *Mol. Microbiol.* 8:143 (1993)). The mutant strain LLO L461T (DP-L4017) was described in Glomski, et al, *J. Cell. Biol.* 156: 1029 (2002), incorporated by reference herein. The *actA* mutant (DP-L4029) is the DP-L3078 strain described in Skoble et al., *J. of Cell Biology*, 150: 527-537 (2000), incorporated by reference herein in its entirety, which has been cured of its prophage. (Prophage curing is described in (Lauer et al., *J. Bacteriol.* 184:4177 (2002); U.S. Patent Publication No. 2003/0203472).) The LLO mutant (DP-L4027) (Lauer et al., *J. of Bacteriology*, 184:4177-4186 (2002)), and LLO  $\Delta$ 26 (DP-L4042) (Decatur et al, *Science* 290:992 (2000)) were also described previously. Construction of an *actA*<sup>-</sup>*uvrAB*<sup>-</sup> strain is described in the copending U.S. provisional application 60/446,051, filed February 6, 2003, as L4029/*uvrAB* (see, e.g. Example 7 of that application). DP-L4029/*uvrAB* (*actA*<sup>-</sup>/*uvrAB*<sup>-</sup>) was deposited with ATCC October 3, 2003, assigned PTA-5563.

[0219] *Construction of pKSV7-dl inlB for deletion of inlB from Listeria by allelic exchange.* Deletion of *inlB* from *Listeria* DP-L4029 (or from other selected mutant strains or from wild-type *Listeria*) can be effected by allelic exchange, as described by Camilli et al., *Mol. Microbiol.* 8:143-147 (1993). Overlapping PCR can be used to prepare the construct used in the allelic exchange procedure. The source of the internalin B gene is the sequence listed as Genbank accession number AL591975 (*Listeria monocytogenes* strain EGD, complete genome, segment 3/12; *inlB* gene region: nts. 97008-98963) and/or the sequence listed as Genbank accession no. NC\_003210 (*Listeria monocytogenes* strain EGD, complete genome, *inlB* gene region; nts. 457008-458963) both of which are incorporated by reference herein in their entirety.

[0220] In the primary PCR reactions, approximately 1000 bps of sequence upstream and downstream from the *Listeria inlB* gene 5' and 3' ends, respectively, are amplified using the following template and primers:

*Template:* DP-L4056 or DP-L4029 genomic DNA

*Primer pair 1* (For amplification of region upstream from 5' end of *inlB*):

Lm-9603 1F: 5'-GTTAAGTTTCATGTGGACGGCAAAG (SEQ ID NO:22) ( $T_m$ : 72°C)

Lm-(3' *inlB*-R +) 97020R: 5'-

AGGTCTTTTTCAGTTAACTATCCTCTCCTTGATTCTAGTTAT  
(SEQ ID NO:23) ( $T_m$ : 114°C)

(The underlined sequence complementary to region downstream of *InlB* carboxy terminus.)

(Amplicon Size (bps): 1007)

*Primer pair 2* (For amplification of region downstream from 3' end of *inlB*):

Lm-(5' *inlB*-F +) 98911F: 5'-

CAAGGAGAGGATAGTTAACTGAAAAAGACCTAAAAAAGA  
AGGC (SEQ ID NO:24) ( $T_m$ : 118°C) (The underlined sequence is complementary to region upstream of *InlB* amino terminus.)

Lm-99970R: 5'-TCCCCTGTTTCCTATAATTGTTAGCTC (SEQ ID NO:25) ( $T_m$ : 74°C)

(Amplicon size (bps): 1074)

[0221] In the secondary PCR reaction, the primary PCR amplicons are fused through overlapping PCR, taking advantage of complementarity between reverse primer from pair 1 and the forward primer of pair 2. This results in precise deletion of *inlB* coding sequence: nts. 97021-98910=1889 bps. The following template and primers were utilized in the secondary PCR reaction:

*Template:* Cleaned primary PCR reactions

*Primer pair:*

Lm-96043F: 5'-GTGGACGGCAAAGAAACAACCAAAG (SEQ ID NO:26) ( $T_m$ : 74°C)

Lm-99964R: 5'-GTTTCCTATAATTGTTAGCTCATTTTTTTC (SEQ ID NO:27) ( $T_m$ : 74°C)

(Amplicon size (bps): 2033)

[0222] A protocol for completing the construction process is as follows:

[0223] The primary PCR reactions (3 temperature cycle) are performed using Vent DNA polymerase (NEB) and 10  $\mu$ l of a washed 30°C *Listeria* DP-L4056 OR DP-L4029 overnight culture. The expected size of *Listeria* amplicons by 1% agarose gel (1007 bps and 1074 bps). The primary PCR reactions are gel purified and the DNA eluted with GeneClean (BIO 101).

[0224] A secondary PCR reaction is performed, utilizing approximately equal amounts of each primary reaction as template (ca. 5 µl). The expected size of the *Listeria* amplicon from the secondary PCR reaction is verified by 1% agarose gel (2033 bps). Adenosine residue are added at the 3' ends of *Listeria* dl inlB amplicon with Taq polymerase.

[0225] The *Listeria* dl inlB amplicon is then inserted into a pCR2.1-TOPO vector. The pCR2.1-TOPO-dl inlB plasmid DNA is digested with XhoI and KpnI and the 2123 bp fragment is gel purified. The KpnI/XhoI 2123 bp fragment is inserted into a pKSV7 vector that has been prepared by digestion with KpnI and XhoI and treatment with CIAP (pKSV7-dl inlB). [Correct?] The fidelity of dl inlB sequence in pKSV7-dl inlB is then verified. The inlB gene is deleted from desired *Listeria* strains by allelic exchange with pKSV7-dl inlB plasmid.

[0226] *Construction of antigen-expressing strains.* Mutant *Listeria* strains expressing a truncated form of a model antigen ovalbumin (OVA), the immunodominant epitope from mouse colorectal cancer (CT26) known as AH1 (SPSYVYHQF; SEQ ID NO:20), and the altered epitope AH1-A5 (SPSYAYHQF; SEQ ID NO:21; Slansky et al., *Immunity*, 13:529-538 (2000)) were prepared. The pPL2 integrational vector (Lauer et al., *J. Bacteriol.* 184:4177 (2002); U.S. Patent Publication No. 2003/0203472) was used to derive OVA and AH1-A5/OVA recombinant *Listeria* strains containing a single copy integrated into an innocuous site of the *Listeria* genome.

[0227] *Construction of OVA-expressing Listeria (DP-L4056).* An antigen expression cassette consisting of hemolysin-deleted LLO fused with truncated OVA and contained in the pPL2 integration vector (pPL2/LLO-OVA) is first prepared. The *Listeria*-OVA vaccine strain is derived by introducing pPL2/LLO-OVA into the phage-cured *L. monocytogenes* strain DP-L4056 at the PSA (Phage from ScottA) attachment site tRNA<sup>Arg</sup>-attBB'.

[0228] PCR is used to amplify the hemolysin-deleted LLO using the following template and primers:

Source: DP-L4056 genomic DNA

Primers:

Forward (*KpnI*-LLO nts. 1257-1276):

5'-CTCTGGTACCTCCTTTGATTAGTATATTC (SEQ ID NO: 28)

(T<sub>m</sub>: LLO-spec: 52°C. Overall: 80°C.)

Reverse (*BamHI*-*XhoI*-LLO nts. 2811-2792):

5'-CAATGGATCCCCTCGAGATCATAATTTACTTCATCCC  
(SEQ ID NO:29)

(T<sub>m</sub>: LLO-spec: 52°C. Overall: 102°C.)

[0229] PCR is also used to amplify the truncated OVA using the following template and primers:

Source: pDP3616 plasmid DNA from DP-E3616 *E. coli* (Higgins et al., *Mol. Molbiol.* 31:1631-1641 (1999)).

Primers:

Forward (*XhoI*-*NcoI* OVA cDNA nts. 174-186):

5'-ATTTCTCGAGTCCATGGGGGTTCTCATCATC (SEQ ID NO:30)

(T<sub>m</sub>: OVA-spec: 60°C. Overall: 88°C.)

-Reverse (*XhoI*-*NotI*-*HindIII*): -

5'-GGTGCTCGAGTCCGCGCCGCAAGCTT (SEQ ID NO:31)

(T<sub>m</sub>: Overall: 82°C.)

[0230] One protocol for completing the construction process involves first cutting the LLO amplicon with *KpnI* and *BamHI* and inserting the *KpnI*/*BamHI* vector into the pPL2 vector (pPL2-LLO). The OVA amplicon is then cut with *XhoI* and *NotI* and inserted into the pPL2-LLO which has been cut with *XhoI*/*NotI*. (Note: The pPL2 vector does not contain any *XhoI* sites; pDP-3616 contains one *XhoI* site, that is exploited in the OVA reverse primer design.) The construct pPL2/LLO-OVA is verified by restriction analysis (*KpnI*-LLO-*XhoI*-OVA-*NotI*) and sequencing. The plasmid pPL2/LLO-OVA is introduced into *E. coli* by transformation, followed by introduction and integration into *Listeria* (DP-L4056) by conjugation, exactly as described by Lauer et al. (or into another desired strain of *Listeria*, such as an *inlB* mutant or an *inlB actA* double mutant).

[0231] A description of the insertion of an antigen expression cassette that expresses OVA can also be found in Example 8 of the U.S. provisional application entitled "Free-Living Microbe Based Vaccine Compositions", US Serial No. 60/511,869, filed October 15, 2003.

[0232] Construction of *Listeria strains* expressing AH1/OVA or AHZ-A5/OVA. To prepare *Listeria* expressing either the AH1/OVA or the AH1-A5/OVA antigen sequences, inserts bearing the antigen are first prepared from oligonucleotides and then ligated into the vector pPL2-LLO-OVA (prepared as described above).

**[0233]** The following oligonucleotides are used in preparation of the AH1 or AH1-A5 insert:

*AH1 epitope insert (ClaI-PstI compatible ends):*

Top strand oligo (AH1 Top):

5'-CGATTCCCCTA GTTATGTTTACCACCAATTTGCTGCA (SEQ ID NO:32)

Bottom strand oligo (AH1 Bottom):

5'-GCAAATTGGTG GTAAACATAACTAGGGGAAT (SEQ ID NO:33)

*AH1-A5 epitope insert (ClaI-AvaII compatible ends):*

The sequence of the AH1-A5 epitope is SPSYAYHQF (SEQ ID NO:21)

(5'-AGT CCA AGT TAT GCA TAT CAT CAA TTT-3' (SEQ ID NO:34)).

Top: 5'-CGATAGTCCAAGTTATGCATATCATCAA TTTGC (SEQ ID NO:35)

Bottom: 5'-GTCGCAAATTGATGATATGCATAACTTGGACTAT (SEQ ID NO:36)

**[0234]** The oligonucleotide pair for a given epitope are mixed together at an equimolar ratio, heated at 95 °C for 5 min. The oligonucleotide mixture is then allowed to slowly cool. The annealed oligonucleotide pairs are then ligated at a 200 to 1 molar ratio with pPL2-LLO/OVA plasmid prepared by digestion with the relevant restriction enzymes. The identity of the new construct can be verified by restriction analysis and/or sequencing.

**[0235]** The plasmid can then be introduced into *E. coli* by transformation, followed by introduction and integration into *Listeria* (DP-L4056) by conjugation, exactly as described by Lauer et al. (or into another desired strain of *Listeria*, such as an *inlB* mutant or an *inlB actA* double mutant).

## EXAMPLE 20

Assessment of *in vivo* cytotoxic activity in mice vaccinated with *Listeria monocytogenes*.

**[0236]** A series of studies were done to assess the ability of vaccinated mice to lyse antigen specific target cells *in vivo*. In the first study, Balb/c mice were vaccinated either intravenously (IV) with *Listeria monocytogenes* strains DP-L4029 (*actA*<sup>-</sup>), DP-L4029 expressing AH1/A5, and DP-L4029 *uvrAB*<sup>-</sup> expressing AH1/A5. The AH1/A5 expressing strains were also treated with S-59 UVA according to the second method of

Example 13. The *Listeria* constructs expressing AH1-A5 also express LLO and OVA. Vaccinations were done on day 0, for all groups and additionally on days 1 and 2 for the S-59 treated strains at the dose (0.1 LD<sub>50</sub>) indicated in Table 20. For each strain and control, two groups of 3 mice were vaccinated. A target cell population was prepared by harvesting the spleens of 20 naïve Balb/c mice in RPMI 1640 medium. The cells were dissociated and the red cells lysed. The white blood cells were counted and split into four equal populations. Each group was pulsed with a specific peptide, either target AH1 (SPSYVYHQF (SEQ ID NO:20), from SynPep, Dublin, CA), target AH1-A5 (SPSYAYHQF (SEQ ID NO:21), SynPep), or two populations of control ( $\beta$ -gal, TPHPARIGL (SEQ ID NO:37)), at 0.5  $\mu$ g/mL for 90 minutes at 37 °C. Cells were then washed 3 times in medium, and twice in PBS + 0.1% BSA. Cells were resuspended at  $1 \times 10^7$  per mL in warm PBS + 0.1% BSA (10 mL or less) for labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR). To the target cell suspensions, 1.25  $\mu$ L of a 5mM stock of CFSE was added and the sample mixed by vortexing. To the control cell suspensions, a ten-fold dilution of the CFSE stock was added and the sample mixed by vortexing. The cells were incubated at 37 °C for 10 minutes. Staining was stopped by addition of a large volume (>40 mL) of ice-cold PBS. The cells were washed twice at room temperature with PBS, then resuspended and counted. Each cell suspension was diluted to  $50 \times 10^6$  per mL, and 100  $\mu$ L of each population was mixed and injected via the tail vein of either naïve or vaccinated mice on day 6. For each strain or control, the group of 3 mice was injected with  $\beta$ -gal and AH-1 or  $\beta$ -gal and AH1-A5. After 12-24 hours, the spleens were harvested and a total of  $5 \times 10^6$  cells were analyzed by flow cytometry. The high (target) and low (control) fluorescent peaks were enumerated, and the ratio of the two was used to establish the percentage of target cell lysis relative to the HBSS control population. The results are shown in Table 20 and Figure 23A. (The tables in this example show averages for three mice, while the figures are representative histograms from individual mice for the indicated samples (not necessarily the same mouse).) The vaccination using the S-59 treated strains shows a slightly better response to AH1 for the *uvrAB* mutant and a significantly higher response to AH1-A5 for the *uvrAB* mutant relative to the S-59 treated *actA* strain.

Table 20 *In vivo* cytotoxicity of Balb/c mice vaccinated as indicated on day 0, also days 2 and 3 for S-59 treated strains.

Immunization	S-59	Vaccination dose	% of target cells killed	
			AH1	AH1-A5
HBSS	-	100 $\mu$ L	0	0
<i>actA</i> <sup>-</sup>	-	$5 \times 10^6$	3.8	7.2
<i>actA</i> <sup>-</sup> AH1-A5	-	$5 \times 10^6$	17.9	77.2
<i>actA-uvrAB</i> AH1-A5	-	$5 \times 10^6$	33.6	85.1
<i>actA</i> <sup>-</sup> AH1-A5	+	$1 \times 10^7$ each day	7.1	3.9
<i>actA-uvrAB</i> AH1-A5	+	$1 \times 10^7$ each day	8.7	56.1

[0237] This study was repeated with an additional vaccination at day 14 for all groups and additionally days 15 and 16 for the S-59 treated strains. The labeled target cells were injected on day 20. The results are shown in Table 21 and Figure 23B. The response to the S-59 treated *uvrAB*<sup>-</sup> mutant can be significantly improved with a boost vaccination, this is not the case for the S-59 treated *actA*<sup>-</sup> strain.

Table 21 *In vivo* cytotoxicity of Balb/c mice vaccinated as indicated on day 0 and 14, also days 2, 3, 15, and 16 for S-59 treated.

Immunization	S-59	Vaccination dose	% of target cells killed	
			AH1	AH1-A5
HBSS	-	100 $\mu$ L	0	0
<i>actA</i> <sup>-</sup>	-	$5 \times 10^6$	1.4	-5.9
<i>actA</i> <sup>-</sup> AH1-A5	-	$5 \times 10^6$	27.4	96.4
<i>actA-uvrAB</i> AH1-A5	-	$5 \times 10^6$	52.9	97.0
<i>actA</i> <sup>-</sup> AH1-A5	+	$1 \times 10^7$ each day	3.6	5.7
<i>actA-uvrAB</i> AH1-A5	+	$1 \times 10^7$ each day	19.2	84.5

[0238] A similar study was done using *actA*<sup>-</sup>, *actA*<sup>-</sup> expressing OVA and *actA-uvrAB* expressing OVA, including with and without S-59 treatment for the OVA expressing strains. This study used C57Bl/6 mice. Groups of 6 mice were vaccinated day 0 (also 1 and 2 for S-59 treated) and three of each group was injected with labeled target cells on day 6. The remaining mice were vaccinated day 14 (also 15 and 16 for S-59 treated) and injected with labeled target cells on day 20. In this study, the naïve target



spleen cells were pulsed with  $\beta$ -gal (low CFSE) or SL8 (high CFSE). The results are shown in Table 22 and Figure 23C. Again, the response to the S-59 treated *uvrAB*<sup>-</sup> mutants is enhanced significantly with a boost vaccination.

Table 22 *In vivo* cytotoxicity of Balb/c mice vaccinated as indicated.

Immunization	S-59	Vaccination dose	% of target cells killed	
			primary	boost
HBSS	-	100 $\mu$ L	0	0
<i>actA</i> <sup>-</sup>	-	1 x 10 <sup>7</sup>	-6.6	0.1
<i>actA</i> <sup>-</sup> OVA	-	1 x 10 <sup>7</sup>	98.9	97.1
<i>actA-uvrAB</i> <sup>-</sup> OVA	-	1 x 10 <sup>7</sup>	99.5	98.1
<i>actA</i> <sup>-</sup> OVA	+	1 x 10 <sup>8</sup> each day	0	0
<i>actA-uvrAB</i> <sup>-</sup> OVA	+	1 x 10 <sup>8</sup> each day	46.5	84.8

#### EXAMPLE 21

S-59/UVA treatment of *Bacillus anthracis* with and without *uvrAB* deletion.

[0239] The allelic exchange methods detailed in Examples 7- 9 and Camilli et al., *Molecular Micro.*, 8:143-147 (1993) were used to modify the *Bacillus anthracis* Sterne strain. The virulence of this strain is attenuated (pXO1<sup>+</sup>, pXO2<sup>-</sup>).

[0240] The *uvrAB* gene from *Bacillus anthracis* was identified (Genbank accession number AE017040, *Bacillus anthracis* Ames strain, section 17 of 18 of the complete genome, *uvrAB* genes coding sequence: nts. 212613-217471) and a plasmid based on pKSV7 with the *uvrAB* gene deletion was constructed (pKSV7-*dl uvrAB*) using Splice Overlap Extension (SOE) PCR and the steps described below:

[0241] *Primary PCR reactions:* Approximately 1000 bps of sequence upstream and downstream from the *B. anthracis uvrAB* genes 5' and 3' ends, respectively, were amplified.

Template: *B. anthracis* Sterne genomic DNA

Primer pair 1: Amplification of region 1000 bp upstream from 5' end of *uvrB*.

(Amplicon Size (bps): 1029)

Ba-225099F: 5'-CTGTGC TTTGCGAATGGAAAGAAGC (SEQ ID NO:38) (T<sub>m</sub>: 74°C)

Ba-(3' *uvrA*-R +) 226109R:

5'-GTTTTCATTCATACACTTAGACAAGCGTTGGCTTTTGCA  
CTTC (SEQ ID NO:39) ( $T_m$ : 120°C) (Underlined sequence is  
complementary to region downstream of *uvrA* carboxy terminus.) or  
Ba-226109R: 5'-GACAAGCGTTGGCTTTTGCACTTC (SEQ ID  
NO:40) ( $T_m$ : 72°C).

Primer pair 2: Amplification of region downstream from 3' end of *uvrA*.

(Amplicon size (bps): 990)

Ba-(3' *uvrA*-R +) 230779F:

5'-CAAAAGCCAACGCTTGTCTAAGTGTATGAATGAAAACC  
GAGTGG (SEQ ID NO:41) ( $T_m$ : 126°C) (Underlined sequence is  
complementary to region upstream of *uvrB* amino terminus.) or Ba-  
230779F: 5'-AAGTGTATGAATGAAAACCGAGTGG (SEQ ID  
NO:42) ( $T_m$ : 70°C)

Ba-231769R: 5'-CATATAAAGGTTCCACAATTGCCTTTTC (SEQ ID  
NO:43) ( $T_m$ : 76°C)

[0242] *Secondary PCR reaction:* Fusion of primary PCR amplicons through SOE  
PCR, taking advantage of complementarity between reverse primer of pair 1 and the  
forward primer of pair 2. Results in precise deletion of *uvrAB* coding sequence: nts.  
226110-230779=4670 bps.

Template: Cleaned primary PCR reactions

Primer pair: (Amplicon size (bps): 1973)

Ba-225118F: 5'-GAAGCAGAAATGAAGCCAATACTCAATC (SEQ ID  
NO:44) ( $T_m$ : 78°C)

Ba-231761R: 5'-GGTTCACAATTGCCTTTTCAATAATC (SEQ ID  
NO:45) ( $T_m$ : 74°C)

[0243] *Construction:* Primary PCR reactions (3 temperature cycle) are performed  
using Vent DNA polymerase (NEB) and Sterne strain genomic DNA. Four primary PCR  
reactions are performed both with and without primers used for splice overlap extension  
(SOE). (If reactions containing Ba-(3' *uvrA*-R +) 226109R or Ba-(3' *uvrA*-R +) 226109R  
primers did not yield significant amplicon product, then these primers on amplicons from  
reactions with Ba-225099F/Ba-226109R or Ba-230779F/ Ba-231769R primer pairs were  
used.) The expected size of *anthracis* primary amplicons by 1% agarose gel (1029 bps and

990 bps) was verified. The reaction was cleaned with S6 columns (BioRad) or GeneClean (BIO 101).

[0244] The secondary PCR reaction was performed, utilizing approximately equal amounts of each primary reaction as template (ca. 5  $\mu$ l) were performed. The expected size of the *Listeria* amplicon from secondary PCR reaction by 1% agarose gel (1973 bps) was verified.

[0245] The *anthracis dl uvrAB* amplicon was inserted into pCR2.1-Blunt II-TOPO vector. The plasmid pCR2.1-TOPO-*dl uvrAB* plasmid DNA was digested with *KpnI* and *PstI* and gel-purify 2033 bp fragment. The *KpnI/PstI* 2033 bp fragment was inserted into pKSV7 vector, that had been prepared by digestion with *KpnI* and *PstI* and treatment with CIAP (pKSV7-*dl uvrAB*). The fidelity of *dl uvrAB* sequence in pKSV7-*dl uvrAB* was verified.

[0246] The *uvrAB* genes were deleted from *B. anthracis* Sterne by allelic-exchange with pKSV7-*dl uvrAB* plasmid. The plasmid pKSV7-*dl uvrAB* was introduced into the *B. anthracis* Sterne strain by electroporation selecting for chloramphenicol resistance. The electroporation was done using a freezing step that significantly increased the frequency of electroporation. *B. anthracis* culture was grown O/N in 3 ml BHI 0.5% glycerol shaking at 37°C. 0.5 ml culture was transferred to 50 ml BHI 0.5 % glycerol (OD<sub>600</sub>=0.1) in 500 ml E-flask. The sample was incubated at 200 rpm 37°C. (or 0.1-0.2 ml to 25 ml BHI 0.5% glycerol in 250 ml flask). At OD<sub>600</sub>= 0.6-0.8 (approx 1 hour 45 min), bugs were collected in 500 ml disposable sterile filter apparatus. The bugs were washed 3 x 25 ml each with cold electroporation buffer (1 mM HEPES 10% glycerol pH 7.4). The cells were resuspended in 1/20 original volume (2.45 ml of e-poration buffer for 50 ml culture) and kept on ice. 1  $\mu$ g (1 to 5  $\mu$ l of miniprep) of "very clean" unmethylated plasmid DNA to 0.2 ml cells suspension in a 0.2 cm gap e-poration cuvette (control=no DNA). The sample was then kept on ice for 15 min. The cells were then pulsed at 25  $\mu$ FD, 200 $\Omega$ , 2.5 kV (or, alternatively, 0.4 ml cells were pulsed in 0.4cm cuvette at 400 $\Omega$ ). Time constant was approximately 4-5 msec. Immediately after pulse, 1 ml BGGM (BHI containing 10% glycerol, 0.4% glucose and 10 mM MgCl<sub>2</sub>) was added. The cells are transferred to a sterile polyprop. tube and incubated 37°C 1 1/2 hour, shaking. The cells are pelleted, resuspended in 200  $\mu$ l BGGM and plated on selective media.

[0247] The pKSV7-*dl uvrAB* was integrated into the *B. anthracis* chromosome at 41°C. pKSV7-*dl uvrAB* was allowed to excise and cure at the permissive temperature,

resulting in chloramphenicol sensitive colonies. PCR primers were designed to detect the deletion on the chromosome. 20% of the chloramphenicol sensitive colonies harbored the deletion in the *B anthracis* chromosome. PCR analysis of the *uvrAB*<sup>-</sup> strain indicated retention of the pXO1 virulence plasmid.

[0248] Two *uvrAB*<sup>-</sup> clones (clone 8 and clone 32A) were S-59-treated, along with the parent strain, by growing in BHI at 37 °C at 300 rpm to an OD<sub>600</sub> of 0.3, at which point 50 mL of solution was transferred to a clean flask and S-59 was added to the concentrations indicated in Table 23. These samples were incubated at 37 °C at 300 rpm with vigorous shaking for approximately 1 hour (OD<sub>600</sub> approximately 1.0, approximately 1 x 10<sup>9</sup>/mL). A 1 mL aliquot was removed to assess the titer and the remaining was transferred to a 150 mm Petri dish and irradiated at a UVA dose of 6 J/cm<sup>2</sup> (FX-1019), resulting in a six-log reduction in titer, as compared to the parental strain, as indicated in Table 23, below, and Figure 24. This demonstrates a sensitivity to psoralen treatment in *B. anthracis* that is similar to that observed for *Listeria monocytogenes uvrAB*<sup>-</sup> strains.

Table 23 Attenuation of *Bacillus anthracis* Sterne strain vs. *uvrAB*<sup>-</sup> mutant with psoralen S-59/UVA treatment.

S-59 nM	Bacterial log titer			Log attenuation		
	Sterne	<i>uvrAB</i> <sup>-</sup> (1)	<i>uvrAB</i> <sup>-</sup> (2)	Sterne	<i>uvrAB</i> <sup>-</sup> (1)	<i>uvrAB</i> <sup>-</sup> (2)
0	8.26	8.13	8.31	-	-	-
25	-	7.46	7.45	-	0.67	0.86
50	-	6.31	6.28	-	1.82	2.03
100	-	3.11	3.68	-	5.02	4.63
200	6.84	1	1	1.42	>7.13	>7.31
400	-	1	1	-	>7.13	>7.31
500	5.29	-	-	2.97	-	-
1000	3.11	1	1	5.15	>7.13	>7.31
1500	1	-	-	>7.26	-	-
2500	1	-	-	>7.26	-	-
5000	1	-	-	>7.26	-	-

## EXAMPLE 22

Use of vaccines of the invention for the *in vivo* treatment of human cancers.

[0249] As an example of the treatment or prevention of a human cancer, a vaccine comprising an antigen-presenting cell that has been loaded and activated by infection by a microbe modified so that the proliferation of the microbe is attenuated, wherein the microbial gene expression is substantially unaffected, is administered to an individual. The microbe can be prepared following the protocols of examples 4 and 5, wherein any desired prokaryotic expression cassettes encoding human tumor antigen(s) are incorporated into the microbe, by utilizing, for example the pPL2 integration vector described in Example 8, or any modifications thereof, or by any methods that are common to those in the art. Antigen-presenting cells (APCs) are then loaded and activated with the modified microbes using methods such as those outline herein.

[0250] The resulting APC vaccine may be formulated in crude, or preferably purified form. The vaccine composition may be prepared as a liquid suspension. In addition, they may be formulated with additives such as preservatives (e.g. thimerosal, 2-phenoxy ethanol), stabilizers (e.g. lactose, monosodium glutamate), adjuvants (e.g. aluminum hydroxide, aluminum phosphate, cytokines), antibiotics (e.g. neomycin, streptomycin) or other substances. Formulations may be resuspended or diluted in a suitable diluent such as sterile water, saline, isotonic buffered saline (e.g. phosphate buffered to physiological pH), or other suitable diluent.

[0251] The vaccine may be administered by a variety of routes, including oral, nasal, intravenous, intradermal, intraperitoneal, intramuscular, intralymphatic and subcutaneous routes, as well as by any route that is relevant for any given malignant or infectious disease. An effective amount of the vaccine will be administered to an individual for treatment. For a therapeutic treatment, an effective amount is a dose that will result in the desired immune response, wherein the immune response either slows the growth of the targeted tumors, reduces the size of the tumors, or preferably eliminates the tumors completely. The administration of the vaccine may be repeated at appropriate intervals, and may be administered simultaneously at multiple distinct sites in the vaccinated individual. For the prophylactic treatment, an effective amount is a dose that will result in a protective immune response such that the likelihood of an individual to develop the cancer is significantly reduced. The vaccination regimen may be comprised of a single dose, or may be repeated at suitable intervals until a protective immune response is established.

[0252] The therapeutic treatment of an individual may be started on an individual who has been diagnosed with a cancer as an initial treatment, or may be used in

combination with other treatments. For example, individuals who have had tumors surgically removed or who have been treated with radiation therapy or by chemotherapy may be treated with the vaccine in order to reduce or eliminate any residual tumors in the individual, or to reduce the risk of a recurrence of the cancer. The prophylactic treatment of an individual would be started on an individual who has an increased risk of contracting certain cancers, either due to environmental conditions or genetic predisposition.

### EXAMPLE 23

Generation of recombinant tumor Ag-secreting vaccines based on attenuated strains of *Listeria*.

[0253] Chicken ovalbumin (OVA) fused with a truncated form of Listeriolysin O (LLO) to facilitate antigen secretion and MHC class I processing was used as a model antigen in studies to evaluate the immunogenicity of selected attenuated *Listeria* strains. The tumor antigen expression cassette was incorporated site-specifically into an innocuous site on the chromosome of a panel of attenuated *Listeria* strains with the proprietary pPL2 integration vector. The recombinant *Listeria* strains expressed and secreted the predicted modified LLO-OVA fusion protein as determined by Western blot analysis (data not shown). The growth of each of these recombinants in liquid broth culture as well as the intracellular growth kinetics was also indistinguishable from its parent. Furthermore, the recombinant OVA-expressing strains were shown to have an IV LD<sub>50</sub> that was within a factor of two of the unmodified parental strains (Table 24).

**Table 24. Selected strains of *Listeria monocytogenes***

Strain	Genotype	Phenotype	LD <sub>50</sub> of parental strain	LD <sub>50</sub> of OVA-expressing strain	Fold attenuation / Parent strain
DP-L4056	10403S phage cured	Wild-type	5 x 10 <sup>4</sup>	1 x 10 <sup>5</sup>	2
DP-L4029	$\Delta actA$	In-frame deletion of actA ( <i>actA</i> ) gene; strain grows intracellularly, but does not spread from cell-to-cell	1 x 10 <sup>8</sup>	1 x 10 <sup>8</sup>	1
L4029-uvrAB	$\Delta actA$ ; $\Delta uvrAB$	In-frame deletion of actA ( <i>actA</i> ) gene and uvrAB ( <i>uvrAB</i> ) genes; strain grows intracellularly, but does not spread from cell-to-cell; enhanced susceptibility to DNA damage such as psoralen-induced DNA cross linking	1 x 10 <sup>8</sup>	1 x 10 <sup>8</sup>	1

[0254] The integration vectors facilitate rapid derivation of multiple recombinant *Listeria* vaccine candidates. A single construct can be mated in parallel into any number of unique genetic backgrounds to rapidly create isogenic strains.

## EXAMPLE 24

Psoralen-induced DNA crosslinking to produce non-viable but metabolically active *Listeria*.

[0255] To ensure safety for a *Listeria*-based *ex vivo* antigen delivery platform, in addition to using genetically attenuated *Listeria*, the inventors have engineered a *Listeria* strain that can be fully inactivated through treatment with psoralen, yet is metabolically active and thus retains its ability to infect cells, escape from the phagolysosome and promote the presentation of encoded antigens via the class I pathway. The engineered *Listeria* strain is exquisitely sensitive to inactivation with psoralens, a group of compounds that form irreversible crosslinks in the genomes of bacteria after illumination with UVA light, so that they are incapable of multiplying. Mutant strains of *Listeria* unable to repair psoralen-mediated DNA damage were created by deleting the ultraviolet light resistance (uvr) AB gene (*uvrAB*), which is required for nucleotide-excision repair in *Listeria* and other bacteria (Sancar et al., *Ann. Rev. Biochem.*, 57:29-67 (1988)). The psoralen S-59 is one of a number of Cerus compounds used in the DNA crosslinking technology known as Helinx (Lin, L., Psoralen photochemical treatment of platelets, *Science and Medicine*, 1998; Hei, et al., *Transfusion*, 39:239-48 (1999)). At a psoralen concentration that inactivates the *Listeria uvrAB* deletion mutants to the limit of detection, the parental, non-mutant strain having intact DNA repair mechanisms is more than four logs less sensitive to UVA light inactivation (Figure 25B). S-59/UVA inactivated *Listeria uvrAB* maintained their mitochondrial activity as determined in a MTT assay and retained their capacity to express their genomic repertoire, as determined by <sup>35</sup>S-methionine-labeled pulse-chase experiments (Figure 26 and Figure 10). S-59/UVA inactivated *Listeria uvrAB* but not the inactivated parental strain demonstrated continued expression of their genetic repertoire. The expression level of the inactivated parental strain was significantly diminished, indicating that S-59/UVA treatment at the S-59 concentrations required for full inactivation significantly decreases expression of *Listeria* gene products, including likely the expression of an encoded tumor antigen.

## EXAMPLE 25

Non-viable *Listeria uvrAB* retain the ability to infect DC and escape from the phagolysosome.

[0256] In addition to preserving metabolic activity, it is important to demonstrate efficient antigen loading into the MHC class I pathway by S-59/UVA inactivated *Listeria uvrAB* strains upon infection of dendritic cells (DC). The inventors have now demonstrated that infection of DC with a *Listeria* mutant (DP-L4027) that is unable to escape from the phagolysosome by virtue of deletion of the *hly* gene that encodes LLO eliminates presentation of antigens in context of MHC class I.

[0257] To demonstrate escape from the phagolysosome of *Listeria uvrAB* infected DC, the inventors took advantage of the fact the cytoplasmic *Listeria* are surrounded by host-cell actin filaments, so-called "actin clouds", that can be visualized by fluorescence microscopy. Actin polymerization on the *Listeria* surface is mediated by the bacterial ActA protein and other host cell factors. A murine DC cell line, DC2.4, was infected at multiplicity of infection (MOI) of 1 with *Listeria* wt, S-59/UVA inactivated *Listeria uvrAB* and *Listeria ALLO* (DP-L4027) for 30 minutes at 37°C. Extracellular bacteria were removed carefully by several washes and DC were incubated for an additional 5 hours at 37°C in the presence of gentamicin to prevent the growth of extracellular bacteria. DC2.4 infected with wild-type *Listeria* or fully inactivated *Listeria uvrAB* demonstrated typical actin clouds or actin comet tails, typical for cytoplasmic localization of *Listeria* (Figure 27). However, in DC2.4 cells infected with the *Listeria* LLO null mutant no co-localization of actin and *Listeria* could be observed, indicating that the bacteria were unable to escape from the phagolysosome. This result demonstrated that these DNA repair mutants retain the ability to escape from the phagolysosome and enter the cytosol of the infected cell, where antigen can be secreted, a requisite step for direct presentation via the MHC class I pathway. See also Example 17, above, and Figure 21.

#### EXAMPLE 26

Non-viable *Listeria uvrAB* efficiently load antigen into the MHC class I pathway of infected dendritic cells (DC).

[0258] Due to the unique ability of S-59 psoralen inactivated *Listeria uvrAB* to escape the phagolysosome within the infected cell, gene products secreted by cytosolic *Listeria* are processed and presented via the MHC class I pathway. To test the ability of S-59/UVA inactivated *Listeria uvrAB* to load antigen into the MHC class I pathway of DC, DC2.4 cells were infected at a multiplicity of infection (MOI) of 1 with the OVA-



expressing *Listeria* strain, L4029 *uvrAB* OVA, inactivated with different concentrations of S-59. The parental *Listeria* OVA and heat-killed *Listeria uvrAB* OVA served as controls. Presentation of OVA peptide by DC2.4 on class I molecules following phagocytosis of *Listeria* was measured after incubation with B3Z cells. B3Z is a LacZ-inducible CD8<sup>+</sup> T-cell hybridoma specific for the OVA<sub>257-264</sub> (SL8) epitope presented on the murine K<sup>b</sup> class I molecule (Sanderson, *Int. Immunol.*, 6:369-76 (1994)). Class I-restricted presentation of SL8 to B3Z cells results in the induction of  $\beta$ -gal synthesis by B3Z. The amount of  $\beta$ -gal produced can be measured by the hydrolysis of the chromogenic substrate CPRG and is an indication of the amount of SL8/ K<sup>b</sup> complexes presented on the surface of APCs. As shown in Figure 9A and 9B, S-59/UVA inactivated *Listeria uvrAB* OVA strain, but not the cognate parental strain, maintained its capacity to load antigen into the MHC class I pathway independent of its ability to multiply. (This is the same data as described in Example 11.) Even at full inactivation using S-59 concentrations of 70 to 100 nM, more than 90% of B3Z activation was maintained. In contrast, the parental *Listeria* OVA strain with intact DNA repair lost its ability to activate the B3Z T-cell hybridoma when higher concentrations of S-59 were used for inactivation. In contrast to the *Listeria uvrAB* OVA strain, B3Z activation and the ability of the parental *Listeria* OVA strain to form colonies on BHI agar plates was closely correlated, suggesting that only viable *Listeria* OVA are capable of infecting DC2.4 cells and loading antigen into the MHC class I pathway. Moreover, heat-killed *Listeria uvrAB* OVA did not result in B3Z activation. This result demonstrates the capacity of *Listeria* to load antigen into the MHC class I pathway can be unlinked from the requirement for multiplication using S-59/UVA inactivated *Listeria*, modified to prevent their capacity to repair psoralen-mediated DNA damage.

[0259] To test the ability of *Listeria uvrAB* OVA to load antigen into the MHC class I pathway of primary DC, immature murine BM-DC were infected with fully inactivated S-59/UVA treated *Listeria uvrAB* OVA. Viable *Listeria uvrAB* OVA, the parental strain and L4027 served as controls. As shown in Figure 28, BM-DC infected with the OVA-expressing but not the parent strains stimulated the B3Z cells *in vitro*. No significant difference between the live and non-viable S-59/UVA treated *Listeria uvrAB* mutant strain (L4029 *uvrAB* OVA) was observed, suggesting that MHC class I molecules of primary DC are efficiently loaded with *Listeria*-derived peptides following the escape of the bacteria from the phagolysosome into the cytosol in spite of the inability of *Listeria uvrAB* to multiply. Importantly, *Listeria actA* OVA inactivated by heat-killing did not result in any significant presentation of OVA peptides in the MHC class I pathway

suggesting that incubation of DC with heat-killed bacteria does not result in any significant antigen loading of MHC class I molecules.

#### EXAMPLE 27

*Listeria* directly infect and activate human DC.

[0260] For the development of a potent antigen delivery platform it is widely thought that activation/maturation of DC is required in addition to efficiently delivering antigen into the MHC class I pathway. In situ, immature DC reside in peripheral tissues where they continuously take up and process antigen, but it is the encounter of an activation stimulus, such as that which bacteria provide, that initiates the activation/maturation process, leading to modulation of chemokine receptors and migration of DC to the T cell area of the draining lymph node. We assessed the potency of wild-type *Listeria* (L4056) to induce phenotypic maturation and cytokine production of human monocyte-derived DC. As shown in Figure 29, encounter of human immature DC with *Listeria* led to up-regulation of the activation markers, CD86 and HLA-DR (Fig. 29A), as well as the maturation marker, CD83 (Fig. 29B). Furthermore, the exposure of human immature DC to *Listeria* increased their immune-stimulatory capacity as shown by their ability to secrete high levels of pro-inflammatory cytokines, such as IL-12p70 and TNF- $\alpha$  (Fig. 29C).

#### EXAMPLE 28

S-59/UVA inactivated *Listeria uvrAB* OVA induce OVA-specific immunity *in vitro*.

[0261] We assessed the potency of the S-59/UVA inactivated *Listeria uvrAB* OVA vaccine to induce OVA-specific immunity *in vivo*. Female C57BL/6 mice were vaccinated intravenously with  $1 \times 10^8$  particles of S-59/UVA inactivated *Listeria uvrAB* OVA. The induction of OVA-specific immunity was assessed 7 days post vaccination. Strikingly, mice that received S-59/UVA inactivated *Listeria uvrAB* OVA but not the parent *Listeria* OVA strain mounted a significant OVA-specific CD8<sup>+</sup> T cell response, as shown in Figure 30. Furthermore, vaccination of mice with heat-killed *Listeria uvrAB* OVA did not result in the induction of OVA-specific immunity.

## EXAMPLE 29

Construction of two recombinant attenuated *Listeria actA/uvrAB* strains expressing full-length CEA containing either the native (CAP1) or the enhanced agonist cytotoxic T lymphocyte epitope (CAP1-6D).

[0262] CEA is a 180 kDa large protein found in adenocarcinomas of endodermally derived digestive system epithelium and fetal colon. CEA is attached to the membrane of cells by a GPI-anchor. The protein contains 7 immunoglobulin-like domains and the C-terminus demonstrates homology with the Non-specific Cross-reacting protein, NCA, a member of the carcinoembryonic antigen gene family. We propose to construct the full-length CEA containing either the HLA\*A0201-restricted CEA native T cell epitope CAP 1 (YLSGANLNL) (SEQ ID NO:51) or the enhancer agonist cytotoxic T lymphocyte peptide CAP1-6D (YLSGADLNL) (SEQ ID NO: 52) (Zaremba et al., *Cancer Res.*, 57:4570-7 (1997)) that has been demonstrated to be more potent at inducing CEA-specific immunity in cancer patients (Table 25) (Fong et al., *Proc. Natl. Acad. Sci. U.S.A.*, 98:8809-14 (2001)).

Table 25.

	Plasmid	Antigen	T cell epitope
1	pPL2 CEA wt	Full-length CEA	CAP1
2	pPL2 CEA-610D	Full-length CEA	CAP1-6D

[0263] The CEA tumor antigen expression plasmids are constructed on the pPL2 backbone, a vector that integrates site-specifically into the *Listeria* genome (Lauer et al., *J. Bacteriol.*, 184:4177-86 (2002)). The two plasmids will be constructed so that the secretion signal and PEST elements derived from *Listeria* LLO are fused genetically with full-length CEA cDNA. Starting from the 5' end of the gene construct, the fusion protein will consist of the N-terminal region of LLO to promote bacterial secretion fused to CEA. Precise linkage of the domains will be accomplished by overlapping PCR. The fidelity of all plasmid constructs will be confirmed by DNA sequencing.

## EXAMPLE 30

Derivation of two attenuated recombinant *Listeria* strains containing pPL2 CEAwt and pPL2 CEA-610D integrated into *Listeria* strain L4029 *uvrAB* ( $\Delta$ actA,  $\Delta$ uvrAB), and verify expression and secretion of CEA antigens.

[0264] Integration of the pPL2-CEA constructs adjacent to the tRNA<sup>Arg</sup> gene in the genome of the *Listeria* strain L4029 *uvrAB* are accomplished as described previously by Lauer et al., *J. Bacteriol.*, 184:4177-86 (2002). Briefly, plasmids are first introduced into *E. coli* strain SM10 by transformation, and then introduced into the desired strain of *Listeria* by conjugation. *Listeria* trans-conjugants are selected by chloramphenicol (pPL2) and streptomycin (*Listeria* strain) selective media; the efficiency of this process is approximately  $1 \times 10^{-4}$ . To ensure purity of trans-conjugants, and to ensure integration of the pPL2 backbone into the bacterial chromosome, a limited number of candidate colonies are passaged three times by streaking onto fresh selective media. Precise integration of the CEA construct into the *Listeria* genome are confirmed by colony-PCR.

[0265] Antigen expression and secretion of the LLO-CEA fusion protein are determined by Western blotting of whole cell lysates, and TCA precipitated bacterial culture fluids. An LLO-specific rabbit polyclonal antibody and a CEA-specific monoclonal antibody are used to verify expression and secretion of the LLO-CEA fusion protein from recombinant *Listeria*. One can compare the biological properties of the recombinant *Listeria* strains expressing CEA to their respective parent strain. The growth kinetics in brain heart infusion (BHI) broth following inoculation by dilution of a stationary phase culture 1:100 into fresh media are determined. In the past we have expressed proteins of similar or larger size in *Listeria*. However, recombinant protein expression of mammalian gene products in bacteria might pose a challenge dependent on each individual protein. If CEA expression in *Listeria* poses a problem, one can construct *Listeria* strains that express either fragments of CEA or the T cell mini-epitope. The HLA\*A0201-restricted CEA native T cell epitope CAP1 (YLSGANLNL) (SEQ ID NO:51) or the enhancer agonist cytotoxic T lymphocyte peptide CAP1-6D (YLSGADLNL) (SEQ ID NO:52) will be embedded in-frame within Ovalbumin (OVA) of our existing expression constructs, whereby the secretion signal and PEST elements derived from *Listeria* LLO are fused genetically with OVA. Expression and immunogenicity of T cell mini-epitopes are conserved as previously demonstrated with the gp70 T cell mini-epitopes, AH1 and AH1-A5 and B16 Trp1, Trp2, and gp100 (data not shown).

#### EXAMPLE 31

Establishment of conditions that fully inactivate *Listeria actA/uvrAB* CEA strains by S-59/UVA treatment, yet retain optimal metabolic activity, tumor antigen expression, infection of antigen presenting cells and phagolysosomal escape.

[0266] Metabolic activity as a result of gene expression is best preserved with a minimal number of crosslinks. One can readily establish conditions for the minimal amount of S-59/UVA treatment that fully inactivates *Listeria actA/uvrAB* CEA vaccines, leaving antigen expression levels intact. An example of inactivation conditions is the addition of S-59 psoralen to 200 nM in a log-phase culture of  $OD_{600} = 0.5$ , followed by inactivation with  $6 \text{ J/m}^2$  of UVA light when the culture reaches an optical density of one. Inactivation conditions are optimized by varying concentrations of S-59, UVA dose, the time of S-59 exposure prior to UVA treatment as well as varying the time of treatment during bacterial growth of the *Listeria actA/uvrAB* CEA strain. The parental *Listeria* strain is used as control. Inactivation of *Listeria* (log-kill) is determined by the inability of the bacteria to form colonies on BHI (Brain heart infusion) agar plates. In addition, one can confirm the expression of CEA and virulence factors, such as LLO and p60, of S-59/UVA inactivated *Listeria* using  $^{35}\text{S}$ -pulse-chase experiments to determine the synthesis and secretion of newly expressed proteins post S-59 / UVA inactivation. Expression of LLO and p60 using  $^{35}\text{S}$ -metabolic labeling can be routinely determined. S-59/UVA inactivated *Listeria actA/uvrAB* CEA will be incubated for 1 hour in the presence of  $^{35}\text{S}$ -Methionine. Antigen expression and secretion of the LLO-CEA fusion protein, endogenous LLO, and p60 will be determined of both whole cell lysates, and TCA precipitation of bacterial culture fluids. LLO-, p60- and CEA-specific monoclonal antibodies will be used for immunoprecipitation to verify the continued expression and secretion from recombinant *Listeria* post inactivation. The expression level of S-59/UVA inactivated *Listeria actA/uvrAB* CEA will be compared to our current *Listeria*-OVA vaccine strain that results in the induction of potent antigen-specific T cell responses. One can select S-59/UVA conditions that lead to reproducible full inactivation with limited affect on expression levels of the assessed gene products.

#### EXAMPLE 32

Establishment of a protocol and vaccine strain for infection of human immature dendritic cells (DC) with inactivated (S-59/UVA) *Listeria actA/uvrAB* CEA vaccines, that results in efficient presentation of CEA in context of MHC Class I.

[0267] Optimal conditions for *ex vivo* infection of DC are determined based on the results of three independent assays: (1) change in phenotype and cytokine profile of human immature DC upon infection, (2) the potency of *Listeria*-infected DC to induce an allogeneic T lymphocyte response, and (3) the potency of *Listeria actA/uvrAB* CEA infected DC to stimulate a CEA-specific HLA\* A0201-restricted T cell line *in vitro*.

[0268] *I. Determination and comparison of the phenotype and cytokine secretion profile of human immature DC infected with live and fully inactivated Listeria-CEA strains. Comparison of the activation of Listeria-infected human DC with commonly used activation signals such as LPS, TNF- $\alpha$ , and  $\alpha$ -CD40.*

[0269] One can characterize and optimize the efficiency of S-59/UVA inactivated *Listeria actA/uvrAB* CEA strains to infect and activate primary human DC. Human DC are enriched from unmobilized peripheral blood as previously described (Fong et al., *J. Virol.*, 76:11033-41 (2002)). Briefly, PBMC are obtained by centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and then monocytes are depleted by density centrifugation through Percoll (Pharmacia) as previously described Mayordomo et al., *Nat. Med.*, 1:1297-302 (1995). Monocyte-depleted PBMC are incubated in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% pooled human AB serum without the addition of exogenous cytokines. After a 24-h culture in a humidified incubator at 37°C with 10% CO<sub>2</sub>, DC are further enriched from lymphocytes by centrifugation through a 15% (w/v) metrizamide gradient (Sigma, St. Louis, MO). The phenotype of the enriched DC population is verified by flow cytometry (HLA-DR expression and lack of CD3, CD14, CD19, and CD56 expression) and dextran uptake. To assess the infectivity of DC with *Listeria*, DC is incubated at different MOI with S-59/UVA inactivated *Listeria actA/uvrAB* CEA strains for one hour. Live *Listeria* will be used as comparison. After extensive washes to remove any extracellular *Listeria*, infected DC is further incubated in the presence of 50  $\mu$ g/mL gentamicin to kill extracellular bacteria. Phenotypic changes upon infection of DC with *Listeria  $\Delta$ actA $\Delta$ uvrAB* CEA strains are assessed by determining cell surface expression of CD80, CD83, CD86, and MHC class II using flow cytometry at different time points post infection. Expression of T helper-1 and T helper-2 type cytokines is measured from the supernatant of infected DC cultures using the Cytometric Bead Array Kit (Pharmingen). Infection and activation conditions are compared to commonly used stimuli such as LPS, TNF- $\alpha$ , and  $\alpha$ -CD40. Infection conditions are

selected that result in potent and consistent stimulation and activation of human DC *in vitro* as well as secretion of cytokines that are most similar to the parental live *Listeria* strain. If the overall infectivity of DC isolated from peripheral blood without the use of cytokines is low, infection of DC prior to the density gradient centrifugation will be assessed.

Moreover, additional sources of DC such as monocyte-derived DC will be assessed for their infectivity for non-viable and live *Listeria*. Briefly, human monocytes are enriched using negative selection and suspended in medium (RPMI-1640+10% FCS) at  $1 \times 10^6$  cells/mL, supplemented with 1000 U/mL GM-CSF and 1000 U/mL IL-4. After 6-7 days of culture, the phenotype of the *in vitro* cultured DC population is verified by flow cytometry and dextran uptake. The phenotypic change as well as the cytokine secretion pattern of monocyte-derived DC upon *Listeria* infection is assessed as described previously.

[0270] 2. Determination and comparison of the stimulatory potency of human immature DC infected with live or fully inactivated *Listeria*-CEA strains to activate allogeneic T cells *in vitro*.

[0271] To address the stimulatory capacity of the *Listeria*-infected DC population, one can determine their ability to stimulate primary allo-reactive T cells in mixed leukocyte reactions (MLR). It is widely believed that the relative potency of an APC to elicit immune responses *in vivo*, which depends on their activation/maturation state, is reflected by their capacity to stimulate an allogeneic T cell response *in vitro* (Jung et al., Immunity, 17:211 (2002)). Briefly, DC are isolated and infected with fully inactivated *Listeria actA/uvrAB* CEA. The phenotype of the infected cell population will be verified by flow cytometry. Various numbers of irradiated (3000 rad) DC are co-cultured with  $5 \times 10^4$  allogeneic responders in 96-well U-bottom plates (Costar, Cambridge, MA). PBMC from random donors are used as responders. After 6 days, the cultures are pulsed with 1  $\mu$ Ci of [ $^3$ H] thymidine for 18 hours. Cells are harvested onto glass fiber sheets and the incorporation of [ $^3$ H] thymidine will be determined by measuring the radioactivity on the scintillation counter. The stimulatory capacity of DC infected with non-viable *Listeria* is compared to DC infected with live *Listeria* as well as DC activated using stimuli such as LPS, TNF- $\alpha$ , and  $\alpha$ -CD40.

[0272] 3. Assessment of the potency of *Listeria*-infected human DC to activate a CEA-specific HLA\*A0201-restricted T cell line *in vitro*. Comparison of immature human DC infected with either live or fully inactivated *Listeria* to peptide-pulsed DC.

[0273] Phenotypic changes, cytokine secretions profile as well as the allo-stimulatory capacity of DC represent an indirect measure for the potency of DC to stimulate an antigen-specific T cell response *in vivo*. The potency of DC to present the recombinant tumor antigen expressed by the fully inactivated *Listeria actA/uvrAB* CEA strain is assessed on the basis of activation of a CEA-specific HLA\*A0201-restricted T cell line generated by L. Fong (unpublished data). Briefly, DC is isolated from peripheral blood of HLA\*A0201 positive donors as described in Milestone 3-1. Various numbers of irradiated (3000 rad) DC, infected under optimal conditions, are co-cultured with  $5 \times 10^4$  CEA-specific HLA\*A0201-restricted T cells in 96-well U-bottom plates (Costar, Cambridge, MA). After 24 hours, cell supernatants are collected. T cell activation is measured on the basis of IFN- $\gamma$ , GM-CSF, or IL-2 secretion. Secreted cytokines are determined using commercially available Cytometric Bead Array kits (Pharmingen). The stimulatory capacity of DC infected with non-viable *Listeria* is compared to DC infected with live *Listeria* as well as DC activated using stimuli such as LPS, TNF- $\alpha$ , and  $\alpha$ -CD40.

### EXAMPLE 33

Confirmation of potency of *Listeria*-loaded primary human DC to prime CEA-specific immunity *in vitro* and select the lead *Listeria* strain for further development.

[0274] To confirm that S-59/UVA inactivated *Listeria*-infected DC are capable of priming naïve CEA-specific CD8<sup>+</sup> T cell response *in vitro*, human immature DC, infected under the established optimal conditions with *Listeria actA/uvrAB* CEA, are used to stimulate naïve T cells *in vitro*. The lead *Listeria actA/uvrAB* CEA strain containing either the native or altered T cell epitope is selected based on its potency to induce naïve CEA-specific T cell responses as determined by three independent assays: (1) [<sup>3</sup>H] thymidine incorporation of the DC-primed T cell cultures; (2) the cytotoxic activity of primed CEA-specific T cell cultures, measured in a <sup>51</sup>Cr release assay; and (3) the frequency of CEA-specific T cells determined by peptide:MHC tetramer staining. Optimal infection is confirmed by phenotypic changes of DC, assessed by determining cell surface expression of CD80, CD83, CD86, and MHC class II using flow cytometry, as well as the cytokine profile secreted by the infected DC. For the induction of primary T cell responses, a constant number of CD45RA<sup>+</sup> T lymphocytes ( $2 \times 10^5$ /well) is co-incubated with varying numbers of irradiated (3,000 R) *Listeria*-loaded DC for 7 days in 96-well, round-bottom



microtiter plates. After 6 days, the cultures are pulsed with 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ] thymidine for 18 hours. Cells are harvested onto glass fiber sheets and the incorporation of [ $^3\text{H}$ ] thymidine will be determined by measuring the radioactivity on the scintillation counter (Wallac, Turku, Finland). Furthermore, induction of CEA-specific T cells is assessed in a cytotoxic T cell assay. Briefly,  $5 \times 10^6$  CD45RA $^+$  T lymphocytes are cultured in parallel with irradiated (3,000 R) *Listeria*-loaded DC at a 10:1 ratio in 24-well plates (Costar) at  $5 \times 10^6$  cells/1.5 ml of media. The cytotoxic activity of the T cells is assessed in a standard 4-hour  $^{51}\text{Cr}$ -release assay after 7 days. Briefly, the target cell lines SW403, SW1417, A375, and T2 are incubated in 250  $\mu\text{Ci}$  of [ $^{51}\text{Cr}$ ] for 2 hours. During this labeling step, T2 cells are also incubated without or with the HLA\*0201-restricted target peptides CAP1 and CAP1-6D. The target cell lines are washed three times with RPMI and plated in triplicate with at least 5,000 targets/well in 96-well U-bottom plates (Costar). Effector cells will be co-incubated with the  $^{51}\text{Cr}$ -labeled target cells at the described effector/target ratios. After a 4-h culture, supernatants are harvested and counted in a Microbeta counter (Wallac, Turku, Finland). Percent specific lysis will be calculated by the formula:

$$100\% \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}).$$

Maximum release is determined by lysis of target cells in PBS containing 0.5% Triton X-100 (Sigma). Lastly, one can determine the frequency of CEA-specific T cells post *in vitro* priming using MHC/tetramers presenting CAP1 or CAP1-6D, as described previously (Fong et al., *Proc. Natl. Acad. Sci. U.S.A.*, 98:8809-14 (2001)). Cryopreserved CD45RA $^+$  T cells obtained before *in vitro* priming is analyzed in parallel with the *in vitro* primed T cell cultures. A total of  $1 \times 10^6$  cells will be stained with the corresponding HLA\*A0201 phycoerythrin-labeled MHC/tetramer for 30 min at room temperature. Antibodies to CD8 (used for positive gate) and to CD4, CD14, CD19, and CD56 (negative "dump" gate) are added at the recommended concentrations and incubated for 30 min at 4°C. Following the staining, samples are washed twice and analyzed with four-color flow cytometry. We have established the background for tetramer staining previously. 20 volunteer blood donors were assessed with the same methodology and had 0.30%  $\pm$  0.18% and 0.27%  $\pm$  0.14% to CEA<sub>605-613</sub> and 610D tetramers, respectively (Fong et al., *Proc. Natl. Acad. Sci. U.S.A.*, 98:8809-14 (2001)).

#### EXAMPLE 34

Use of proteinase 3 or PR1 as a heterologous antigen.

[0275] Although some of the procedures outlined in the specific examples above describe the use of CEA antigens as the antigen expressed by the modified *Listeria*, one of ordinary skill in the art will readily recognize that similar procedures may be used to prepare a modified *Listeria* that expresses a different antigen, such as a proteinase-3 or a proteinase-3 derived antigen, to infect dendritic cells *in vitro* or *ex vivo* in order to effect loading and activation/maturation. One of ordinary skill in the art will also recognize that the resulting DC vaccines may then be administered to an animal or patient to induce an immune response to proteinase-3 and/or PR1.

[0276] For instance, the L4029-*uvrAB* *Listeria* strain described in the Examples above may be modified with a vector comprising a pPL2 vector backbone or the like encoding the proteinase-3 gene and/or the PR1 epitope to integrate the antigen-expressing sequence into the genome of the *Listeria*. In one example, the PR1 antigen could be expressed as part of a fusion protein such as an LLO-OVA/PR1 fusion protein comprising a truncated LLO sequence fused to OVA in which the PR1 epitope has been embedded. The sequence of such an antigenic protein (LLO-OVA/PR3) that could be expressed by the modified *Listeria* is shown in Figure 31.

#### EXAMPLE 35

Measuring the ability of mutant *Listeria* to escape the phagolysosome and promote Class I antigen presentation

[0277] An exemplary protocol for assessing the ability of a particular candidate mutant *Listeria* to escape the phagolysosome of an antigen-presenting cell and promote Class I antigen presentation by the cell is as follows: First, DC2.4 cells are grown on coverslips. The cells are then infected with the desired *Listeria* strain (MOI=100). At 0.5 hpi, the cells rinsed to wash away free *Listeria*. At 1 hpi, gentamicin is added at 50 µg/mL. At 5 hpi, coverslips are washed and fixed for in 3.5% formaldehyde. The coverslips are blocked, stained with rabbit anti-*Listeria* antibody (Difco), and detected with a goat-anti-rabbit FITC secondary (Vector Labs). Actin is detected with Phalloidin-rhodamine (Molecular Probes). The coverslips are mounted with Vectamount+DAPI (Vector Labs) and examined. See also Example 17 and Example 25, above.

## EXAMPLE 36

Generation of Human Monocyte-Derived Dendritic Cells and Infection with *Listeria* Vaccines

[0278] An outline of an exemplary protocol for generation of human monocyte-derived dendritic cells and infection with *Listeria* vaccines is presented below:

[0279] *Materials:* Human peripheral blood (buffy coat from blood donor preferred); Ficoll-Hypaque (Amersham); dPBS w/o Ca, Mg (MediaTech); RPMI-1640 w/ L-Glutamine (MediaTech); Fetal Bovine Serum, Defined, Heat Inactivated (HyClone); Human GM-CSF (R&D Systems)-stock solution made at 500U/ $\mu$ L and stored at -20°; Human IL-4 (R&D Systems)-stock solution made at 200U/ $\mu$ L and stored at -20°; Costar 24-well plates (Fisher).

[0280] *Monocyte Isolation Media (MIM):* To make Solution 1 (Isoosmotic Percoll), 50 mL of NaCl solution (500 mL dH<sub>2</sub>O, 43.84 g NaCl (1.5M)) is added to 450 mL Percoll and mixed. Solution 2 (PBS/Citrate) is prepared by mixing 1000 mL dH<sub>2</sub>O, 205.6 mg NaH<sub>2</sub>PO<sub>4</sub>\*2H<sub>2</sub>O (1.49mM), 1.30 g Na<sub>2</sub>HPO<sub>4</sub> (9.15 mM), 8.18 g NaCl (139.97 mM), and 3.82g C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>\*2H<sub>2</sub>O (13 mM) and bringing the pH to 7.2. 250 mL of isosmotic percoll is then mixed with 250 mL of PBS/citrate. The solution is sterile filtered and stored at 4°

[0281] *Culture media:* RPMI-1640 w. GlutaMax (Gibco) + 10% Fetal Calf Serum (Defined, heat inactivated FCS from HyClone is used).

[0282] *Methods:* Ficoll and MIM are warmed to room temperature. 20 mL of Ficoll into each of 2 50 mL conical tubes. Blood is diluted 2 fold with dPBS and mixed well. 25 mL of blood is layered on top of Ficoll in each tube. The tubes are centrifuged at 400 xg for 30 minutes at 18-20°.

[0283] The mononuclear interface is carefully harvested from the gradient, and placed into a clean 50 mL tube. The remainder of the tube is filled with dPBS. The tube is centrifuged at 100 xg for 15 minutes. This pellets the lymphocytes and monocytes, but leave the platelets suspended. The supernatant is aspirated. The steps of filling the remainder of the tube with dPBS, centrifuging, and aspirating are repeated two more times, for a total of 3 washes.

[0284] The pellet is resuspended in 20 mL of dPBS. The suspension is layered onto 20 mL of MIM. The sample is centrifuged at 400 xg for 35 minutes at room temperature.

The monocytes are harvested from the interface and transferred into a clean tube containing culture media. If culturing DC to use with bacteria, do not use antibiotics.)

[0285] The sample is centrifuged at 400 xg for 10 minutes and the supernatant aspirated. The pellet is washed 4x in dPBS. After the final wash, cell pellet is resuspended in RPMI-1640 +10% FCS. The sample is then counted on hemacytometer using Trypan Blue or using automated counter. The cell suspension is diluted to  $1 \times 10^6$  cells per mL. For each mL of cell suspension, 500U GM-CSF and 200U IL-4 is added (1  $\mu$ L per mL of each if stocks were made as described above). 1 mL per well is plated into Costar 24-well plates. The plates are placed at 37°C, 5% CO<sub>2</sub>, 100% humidity for 48 hours.

[0286] On second day, feeding media for dendritic cells is made up. This consists of 0.5mL culture media (warm to 37° before use) per well cultured, with 500U/mL GM-CSF and 200U/mL IL-4. 0.5 mL from the top of each well is aspirated and replaced with 0.5 mL of fresh feeding media. Plates are placed at 37°C, 5% CO<sub>2</sub>, 100% humidity for 48 additional hours. Feeding is repeated on day 4. On day 5, cells are ready for use. The cells should always be kept in GM-CSF and IL-4 containing media, or they will revert to macrophages. The dendritic cells are examined phenotypically on the cytometer looking at HLA-DR, CD1a, CD83, and CD86.

[0287] *Listeria Infection of Human DC:*

[0288] The day 5 dendritic cells (DC) are pelleted and resuspended in fresh media with GM-CSF and IL-4 at  $2 \times 10^6$  cells per mL. 500  $\mu$ L of suspension is aliquoted to each well of a 24 well plate. Maturation stimuli or bacteria is added in 500  $\mu$ L. 1  $\mu$ g of LPS is used for maturation control. (1000U of IFN- $\gamma$  or 1  $\mu$ g of sCD40L can be added to augment this response.) For *Listeria* infection, between 10-100 *Listeria* per DC is used. Cells are infected for 1 hour, then extracellular bacteria is washed away and cells are resuspended in media containing 50 $\mu$ g/mL gentamicin. sCD40L can be added to enhance DC survival and promote greater IL-12p70 release. 1000U/mL IFN- $\gamma$  can be added to augment maturation and IL-12p70 secretion. The DC are examined phenotypically on the cytometer looking at HLA-DR, CD1a, CD83, and CD86.

## EXAMPLE 37

Asporagenic *B. anthracis* vaccine strains

[0289] *The spoIIE in-frame deletion.* The *spoIIE* region of *B. anthracis* is identified by homology to the same gene in *B. subtilis*. In order to isolate an in-frame deletion of *B. anthracis* SpoIIE, the *spoIIE* gene is first amplified by PCR and cloned into pCR-Blunt II-TOPO (Invitrogen). Next, most of the *spoIIE* gene is deleted by using the technique of gene splicing by overlap extension (SOE) (Horton et al., *Biotechniques* 8:528-35 (1990)). This in-frame deleted *spoIIE* gene is cloned into the shuttle vector pKSV7, which carries a chloramphenicol-resistance gene and cannot replicate at 42°C (Smith et al., *Biochimie*, 74:705-11 (1992)). pKSV7 containing the deleted *spoIIE* gene is then electroporated into *B. anthracis*, and cells are grown at 42°C in the presence of chloramphenicol to select for strains in which the plasmid has integrated by homologous recombination into the *spoIIE* gene. Further growth at 30°C without chloramphenicol selection allows excision and loss of the plasmid. Chloramphenicol-sensitive strains should be found at about 1%, and about half of them should contain the deleted *spoIIE* allele (Camilli et al., (1993)). The presence of the deletion is confirmed by PCR and Southern blot analyses.

[0290] *The spoIIE/uvrAB double deletion strain.* Starting with the *spoIIE* deletion strain, an in-frame deletion of the *uvrA* and *uvrB* genes is made. Once again, the genes of interest are amplified and cloned into pCR-Blunt II-TOPO. Then we shall delete most of the *uvrA* and *uvrB* genes by the SOE technique. This in-frame deleted *uvrAB* region is cloned into pKSV7, and the construct is electroporated into the *B. anthracis* *spoIIE* deletion strain. Chloramphenicol-resistance is selected at 42°C in order to select for the integration of the plasmid into the *uvrAB* region. Growth at 30°C without drug selection is allowed in order to encourage the growth of segregants that have lost the plasmid. Chloramphenicol-sensitive colonies are picked and tested by PCR for loss of the *uvrAB* region, and that loss will be confirmed by Southern blot analysis.

## EXAMPLE 38

A temperature sensitive *recA* mutant of *B. anthracis*

[0291] To generate a temperature sensitive *recA* mutant of *B. anthracis* which grows well at 30°C and is very sensitive to psoralen at 42°C, a mutation is made in *B.*

*anthracis* which is analogous to the V246M mutation of the temperature sensitive *recA* mutant of *E. coli*, *recA44* (Kawashima et al., 193:288-92 (1984)). To make the *B. anthracis* mutant, the sequence 245KVVKNK250 (SEQ ID NO:46), which is conserved between *E. coli* and *B. anthracis*, is mutated. The V246M mutation is introduced into the cloned *B. anthracis recA* gene by mismatched oligonucleotide mutagenesis, using the Stratagene Quick Change kit. The mutations are confirmed by sequence analysis, and the mutated gene is transferred into pKS V7, in order that they can be introduced into the chromosome of *B. anthracis spoIIIE uvrAB* by allelic exchange. Alternatively, the *recA* gene from the *B. anthracis* strains is deleted and replaced with the *recA44(ts)* allele of *E. coli*. (It is known that *B. anthracis recA* functions in *E. coli* (Ko et al., J. Bacteriol 184:3917-22 (2002)).)

#### EXAMPLE 39

##### Introduction of mutations in the active sites of *B. anthracis* antigens

[0292] The lethal factor mutation H686A inactivates its protease activity, and the edema factor mutations K346Q and K353Q (together) inactivate its adenyl cyclase activity (Brossier et al., Infect. Immun., 68:1781-6 (2000)). These mutations are introduced into *B. anthracis* strains to be used in vaccines, such as the *spoIIIE uvrAB* and *spoIIIE uvrAB recA* strains. The *lef* (lethal factor) and *cya* (edema factor, adenyl cyclase) genes are cloned and mutagenized with the Quick Change kit (Stratagene) to create the mutant genes. The mutant genes are then transferred to pKSV7 and finally introduced into the host pXO1 plasmid by allelic exchange.

#### EXAMPLE 40

##### The use of SOS regulatory sequences for expressing protective antigen at high levels

[0293] Cheo et al (Cheo et al., J. Bacteriol., 175:5907-15 (1993)) have shown that the consensus sequence GAACN4GTTTC (SEQ ID NO:47) defines the LexA repressor site for genes in the SOS response of *B. subtilis*. A similar consensus sequence upstream of the promoters for the *B. anthracis recA* and *uvrAB* genes, which are part of the SOS regulon, is to be located. To make a *B. anthracis* strain that expresses protective antigen at high levels, the protective antigen gene is put under the control of the SOS regulatory sequence and introduced it into *B. anthracis spoIIIE uvrAB* strain, so that treatment with psoralen will

cause high levels of protective antigen to be made. In order to insert this artificial gene into the *B. anthracis* chromosome, an integration vector, such as pPL2, is used (Lauer et al, J. Bacteriol., 184:4177-86 (2002)). The gene of interest, in this case the protective antigen gene under control of a promoter, is inserted in the multicloning site. The plasmid is mated from *E. coli* into *B. anthracis* strains. Since it cannot replicate in gram-positive bacteria, it can only be maintained by integration into the chromosome. The current pPL2 vector contains a phage integrase and phage attachment site from *L. monocytogenes*, and therefore, must be modified by removing the *L. monocytogenes* phage integrase gene and phage attachment site and replacing them with similar elements from a phage of *B. anthracis*, such as gamma phage (Brown et al., J. Infect Dis., 96:34-9 (1955)). Also, the pPL2 vector typically contains chloramphenicol-resistance genes for selection. Since drug resistance genes are undesirable for vaccine work, they are removed. One of the drug resistance genes has been replaced by the gene for D-alanine racemase, which synthesizes D-alanine and allows a D-alanine auxotroph to grow on rich medium without the addition of D-alanine. The other drug resistance gene is replaced by the gene for glutamine synthetase, which synthesizes glutamine and allows growth of a glutamine synthetase mutant bacterium on rich medium without glutamine.

#### EXAMPLE 41

##### Exemplary mutant *B. anthracis* strains

[0294] A variety of different mutant *B. anthracis* strains are prepared using combinations of the methods described in the Examples, above. Exemplary mutant *B. anthracis* strains to be used in vaccine compositions are listed in Table 26.

Table 26. *B. anthracis* strains and candidate vaccines

Strain and/or Genotype	Relevant Characteristics and Phenotype	Use and Vaccine Strain Number
Ames pXO1+/pXO2+	Fully virulent wild-type <i>B. anthracis</i> (Toxigenic and encapsulated)	Initial host strain for construction of all vaccine candidates Production of virulent spores for challenge experiments in mice and guinea pigs
Sterne pXO1+/pXO2-	Toxigenic, non-encapsulated	Production of virulent spores for challenge experiments in mice and guinea pigs
<i>spoIIIE</i> pXO1+/pXO2+	Non-sporogenic Toxigenic, encapsulated	Vaccine strain #1
<i>SpoIIIE/uvrAB</i> pXO1+/pXO2+	Non-sporogenic NER <sup>-1</sup> (Increased S-59/UVA sensitivity) Toxigenic, encapsulated	Vaccine strain #2

<i>SpolIE/uvrAB/recA ts</i> <sup>3</sup> pXO1+/pXO2+	Non-sporogenic NER- (Increased S-59/UVA sensitivity) Toxicogenic, encapsulated	Vaccine strain #3
<i>SpolIE/uvrAB/recA ts</i> pXO1+/pXO2+	Non-sporogenic NER-/conditional HR- <sup>4</sup> (Increased S-59/UVA sensitivity) Toxicogenic, encapsulated	Vaccine strain #4
<i>SpolIE/uvrAB/ pXO1</i> ( <i>lef686/cya346/35</i> ) pXO2+	Non-sporogenic NER- (Increased S-59/UVA sensitivity) Encapsulated Non-toxicogenic (LF/EF functional domains mutated)	Vaccine strain #5
<i>SpolIE/uvrAB/ recA ts/</i> pXO1( <i>lef686/cya346/35</i> ) pXO2+	Non-sporogenic NER-/conditional HR- (Increased S-59/UVA sensitivity) Encapsulated Non-toxicogenic (LF/EF functional domains mutated)	Vaccine strain #6
<i>SpolIE/uvrAB/</i> pXO1 ( <i>lef686/cya346/35</i> ) pXO2+/ Pro <sub>S-59</sub> -PA	Non-sporogenic NER- (Increased S-59/UVA sensitivity) Encapsulated Non-toxicogenic (LF/EF functional domains mutated) S-59 psoralen inducible PA	Vaccine strain #7
<i>SpolIE/uvrAB/ recA ts/</i> pXO1( <i>lef686/cya346/35</i> ) pXO2+/ Pro <sub>S-59</sub> -PA	Non-sporogenic NER-/conditional HR- (Increased S-59/UVA sensitivity) Encapsulated Non-toxicogenic (LF/EF functional domains mutated) S-59 psoralen inducible PA	Vaccine strain #8

<sup>1</sup>NER, nucleotide excision repair

<sup>3</sup>Conditional *recA* strains under the control of a *lacI* repressible promoter will also be derived

<sup>4</sup>HR, homologous recombination

#### EXAMPLE 42

Characterization of protein expression levels, including protective antigen and capsule, in psoralen-inactivated *B. anthracis* strains

[0295] To show that inactivated *B. anthracis* strains can still metabolize, the cells are incubated in minimal medium with bicarbonate (Thorne et al., J. Gen. Microbiol., 17:505-16 (1957)). After such incubation the cells are removed by centrifugation and save the supernatant. The supernatant is subjected to SDS-polyacrylamide gel electrophoresis. After staining with Coomassie Blue, protective antigen stands out, and its presence is confirmed by Western blot analysis (Brossier et al., Infect. Immun. 68:5731-4 (2000)) and by mass spectrometry. In addition, mass spectrometry is used to identify the other proteins that are excreted under these conditions, using the methods described in Lenz et al., Proc. Natl. Acad. Sci. U.S.A., 100:12432-12437 (2003). In order to assess whether polyglutamate capsule is made under these conditions, pXO2, which encodes the genes for capsule synthesis, is introduced into the strains by transduction



and (Green et al., Infect. Immun., 49:291-7 (1985). Capsule is measured by rocket immunoelectrophoresis (Uchida et al., Mol. Microbiol., 23:1229-40 (1997)).

#### EXAMPLE 43

Characterization of the humoral and mucosal responses in Swiss Webster and A/J mice immunized with attenuated *B. anthracis* strains

**[0296]** *Mouse Immunization.* Mice are injected with the S-59/UVA vaccines by the intramuscular (IM) or the subcutaneous (SC) routes to determine which route of immunization results in the best bacterial-specific humoral and cellular responses. Intranasal (IN) immunization of mice is also tested to assess mucosal responses induced by the candidate vaccines. IN immunization with 5 µl of a designated vaccine preparation into each nare of lightly anesthetized mice is performed as described previously: (Boyaka et al., *J. Immunol.*, 170: 5636-43 (2003)) Mice are immunized with 0.1 LD<sub>50</sub> doses of the candidate vaccines. Any of the eight S-59/UVA inactivated vaccine candidates in which a median lethality level is not observed is given at an initial dose of 10<sup>8</sup> particles. Mice that are immunized by more than one route are not injected with a combined dose that exceeds the 0.1 LD<sub>50</sub> dose, or is greater than 10<sup>8</sup> particles. Mice given multiple immunizations receive consistent vaccine doses with all injections. As immunization on three consecutive days with S-59/UVA inactivated *Listeria uvrAB* resulted in increased humoral and cellular immunity as compared to a single immunization, the same strategy is used with the *B. anthracis* strain vaccines. Mice are also given booster immunizations at 14 days and 28 days following the primary immunization.

**[0297]** *Quantification of antibodies to PA, LF, EF, capsule, and whole bacteria.* The mucosal and antibody responses in mice immunized with the various vaccine candidates are characterized. Sera is taken from the retroorbital plexus prior to immunization as well as 1 week after each immunization (We have IACUC approval for a maximum of five retroorbital plexus procedures per mouse with blood sampling not to exceed one per week, and to be taken from alternating eyes). Saliva and nasal washes for measurement of IgA levels are performed at the time of sacrifice one week after the final immunization. The durability of the humoral and mucosal immunity induced by the candidate vaccines at 45 days after the final immunization is also characterized. Humoral and mucosal responses against PA, capsule, and vegetative bacteria (Sterne strain) are determined by enzyme-linked immunosorbent assays (ELISAs), as published previously

(Ballard et al., *Proc. Natl. Acad. Sci. U.S.A.*, 93:12531-4 (1996); Rhie et al., *Proc. Natl. Acad. Sci. U.S.A.*, 100:10925-30 (2003)). Briefly, Immulon 96-well Maxisorp plates (Nalge Nunc) are first coated by 5 µg purified PA, LF, EF, BSA conjugated with poly-γ-D-glutamic acid (PGA) capsule prepared as described previously (Rhie et al., *Proc. Natl. Acad. Sci. U.S.A.*, 100:10925-30 (2003)), or with S-59 psoralen/UVA inactivated bacteria ground under liquid nitrogen using a mortar and pestle in 50 mM carbonate buffer (pH 9.6) at 4°C for 16 h, and blocked with TSTA buffer (50 mM Tris [pH 7.6], 142 mM NaCl, 0.05% sodium azide, 0.05% Tween 20, 2% bovine serum albumin). Serial two-fold dilutions of mouse plasma or mucosal secretions are added to the 96-well plates coated with PA, PGA-BSA, or Sterne respectively. Binding of Abs to the immobilized antigens is determined by incubation with isotype-specific peroxidase goat anti-mouse  $\mu$ ,  $\gamma$ , or  $\alpha$  H chain-specific antibodies from Southern Biotechnology Associates (Birmingham, AL). Biotinylated rat anti-mouse  $\gamma$  1 (clone G1-7.3),  $\gamma$  2a (clone R19-15),  $\gamma$  2b (clone R12-3), or  $\gamma$  3 (clone R40-82) H chain-specific mAbs (BD Pharmingen, San Diego, CA) and streptavidin-conjugated peroxidase are used for IgG Ab subclass analysis (Cole, *J. Bacteriol.*, 107:846-52 (1971); Cole et al., *Basic Life Sci.*, 5B:487-95 (1975)). The colorimetric reaction is developed by addition of ABTS substrate (Sigma-Aldrich, St. Louis, MO). End-point titers are expressed as the reciprocal log<sub>2</sub> dilution giving OD<sub>415</sub> > two standard deviations above those obtained with control, non-immunized mice.

[0298] *Enzyme-linked immunospot (ELISPOT) assay for the detection of Ig-secreting cells.* The frequency of PA-specific Ig-secreting lymphocytes is determined by ELISPOT analysis (Boyaka et al., *J. Immunol.*, 170:5636-43 (2003)). Briefly, spleens or cervical lymph nodes of vaccinated and control mice are rapidly dissected out and placed in ice-cooled RPMI 1640 medium and single cell suspensions are prepared. 96-well PVDF-based plates (BD Biosciences, San Jose) are coated overnight with 2.5 µg/ml purified PA (List Biological Laboratories, Campbell, CA). The plates are washed, blocked for 2 hrs at 37°C with 200 µl complete RPMI, and serial dilutions of cell suspensions are added to 96-well plates. Cells are incubated on the plates for 6 hours at 37°C in 5% CO<sub>2</sub>. Antigen-specific Antibody Forming Cells (AFC) are detected with isotype-specific biotin-labeled anti-mouse  $\mu$ ,  $\gamma$ , or  $\alpha$  H chain-specific antibodies (Southern Biotechnology Associates). After incubation at RT for 2 h, the plates are washed, and goat anti-biotin:1nm Gold conjugate (GAB1; Ted Pella) is added for 1 hour at RT. After extensive washing, 30 µl of the silver substrate (Silver Enhancing Kit; Ted Pella) is added into each well and the spot development is monitored. Spots in each well are counted using an automated ELISPOT

plate reader (CTL, Cleveland). The humoral response is expressed as the number of antibody forming cells per 10<sup>6</sup> spleen or lymph node cells.

[0299] *Toxin Neutralization Assays.* Neutralizing antibodies induced in mice immunized with the vaccine candidates are evaluated for the ability to protect the J774 macrophage cell line from lethal toxin (PA+LF) (Mock et al., *Annu. Rev. Microbiol.*, 55:647-71 (2001); Boyaka et al. (2003); Rhie et al. (2003)). Briefly, J774 cells (ATCC, Manassus, VA) are added to 96-well flat-bottom plates (Nunc) at 5 X 10<sup>4</sup> cells/well and incubated for 12 hours at 37° C in 5% CO<sub>2</sub>. Test serum or mucosal secretions are serially diluted two-fold in TSTA buffer. PA and LF (400 ng/ml PA and 40 ng/ml LF) are added to the antiserum dilutions. After incubation for 1 hour the antiserum/lethal toxin complex mixture is added to the cell suspension and incubated for an additional 5 hours. Cell viability is monitored by the MTT assay (absorbance measured at 540nm). Assays are performed in triplicate with a negative control (normal serum) and a positive control (MAbs, 14B7 and 1G3) (Mikesell et al., *Infect Immun.*, 39:371-6 (1983); Starnbach et al., *Nature*, 9, (2003)) included in each plate. The mean and standard deviation of each triplicate sample dilution is calculated. The endpoint is expressed as the highest serum dilution exhibiting 50% neutralization of the anthrax toxin as compared to normal control serum.

#### EXAMPLE 44

Characterization of the PA-, LF-, and EF-specific CD4<sup>+</sup> T cell-mediated responses in A/J mice vaccinated with modified *B. anthracis*

[0300] *T cell Proliferation.* CD4<sup>+</sup> T cell proliferation are determined from PBMC, spleen and lymph node cells of vaccinated and naïve A/J mice. Spleen and cervical lymph nodes are dispersed to obtain single cell suspensions as previously described (Boyaka et al., *J. Immunol.*, 162: 122-8 (1999); Lillard et al., *J. Immunol.*, 166:162-169 (2001); Little et al., *Infect. Immun.*, 65:5171-5 (1997)). CD4<sup>+</sup> T cells are isolated by negative selection using the Mouse CD4<sup>+</sup> T cell isolation kit from Miltenyi Biotec (Auburn, CA). Purified CD4<sup>+</sup> T cells from individual mouse spleens, from pooled lymph nodes or PBMCs are cultured at 4 x 10<sup>6</sup> cells/ml and stimulated with varying concentrations of PA, LF or EF in the presence of T-cell-depleted, non-dividing syngeneic naïve spleen feeder cells (8 X 10<sup>6</sup> cells/ml) in complete RPMI (RPMI supplemented with 10% FBS, 10mM HEPES, 2 mM L-glutamine, 1mM Sodium Pyruvate, non-essential amino acids, 23.8 mM Sodium

Bicarbonate,  $5 \times 10^{-5}$  M  $\mu$ -Mercapthoethanol, 100 U/ml penicillin and 100 Ug/ml streptomycin). The replication of splenic feeder cells is arrested by brief photochemical treatment with S-59 psoralen. Cultures are incubated for 4 days at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  prior to addition of 0.5  $\mu\text{Ci}$  of tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) for the final 18 to 20 hours. The cells are harvested onto glass fiber sheets and the amount of incorporated thymidine is determined by measuring the radioactivity on the scintillation counter (Wallac, Turku, Finland).

**[0301]** *Analysis of PA-, EF- or LF-induced cytokine responses.*  $\text{CD4}^{+}$  T cells are isolated by negative selection using the Mouse  $\text{CD4}^{+}$  T cell isolation kit from Miltenyi Biotec (Auburn, CA). Purified  $\text{CD4}^{+}$  T cells from spleens or lymph node of individual mice are cultured in round-bottom 96-well plates at  $1 \times 10^5$  cells/well and stimulated with varying concentrations of PA, LF or EF in the presence of T cell-depleted, non-dividing syngeneic naïve spleen feeder cells ( $1 \times 10^5$  cells/well) in complete RPMI. The T cell-depleted spleen feeder cells are arrested by a brief photochemical treatment with S-59. T cell cultures are incubated for 2 days at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Expression of T helper-1 and T helper-2 cytokines is determined from supernatants of antigen-stimulated  $\text{CD4}^{+}$  T cells using the Th1/Th2 Cytometric Bead Array kit (BD Pharmingen, San Diego, CA).

#### EXAMPLE 44

Characterization of the extent of protection against spore and lethal toxin challenge in Swiss Webster and A/J mice at 45 days post last immunization dose with modified *B. anthracis* vaccines

**[0302]** *Protection of mice against lethal toxin challenge.* Mice immunized with selected candidate vaccines are challenged by tail vein injection with lethal toxin, as described previously (Price et al., *Infect. Immun.*, 69:4509-15 (2001); Rhie et al. (2003)). Lethal toxin is prepared by mixing recombinant PA and LF recombinant proteins (List Biological Laboratories, Campbell, CA) as described (Rhie et al. (2003)). The lethal toxin IV  $\text{LD}_{50}$  per mouse is approximately 12  $\mu\text{g}$  of PA mixed with 6  $\mu\text{g}$  of LF. The median lethality in mice of freshly prepared lethal toxin is determined by tail vein injection over a 0.1-10  $\text{LD}_{50}$  dose range of the published values. The protection studies will likely include lethal toxin challenge over a range of 5-10 times the  $\text{LD}_{50}$  dose. In this model, unprotected mice succumb within 24 h. Initially, death by anthrax is confirmed in selected mice by plating blood on tryptic soy agar and incubating overnight at  $37^{\circ}\text{C}$ . Plates are observed

for colonies with 2-3mm typical *anthracis*-like "ground glass" appearance. All mice treated with lethal toxin are monitored daily, and experiments are terminated after 2 weeks and all protected mice are sacrificed.

[0303] *Spore preparation.* Sterne strain spores are prepared as described (Barnard and Friedlander, 1999). Briefly, single colonies are inoculated into 5 ml of FA medium (3.3% tryptone, 2% yeast extract [dialyzed overnight against water], 0.2% L-histidine, 0.8% Na<sub>2</sub>HPO<sub>4</sub>, 0.4% KH<sub>2</sub>PO<sub>4</sub>, 0.74% NaCl) contained in a 100-ml bottle and shaken for 5 h at 37°C. One-tenth-milliliter aliquots are spread on L agar plates, and incubated at 37°C. Bacterial lawns are scraped from the plates, washed extensively with sterile water, heat shocked for 30 min at 60°C, washed with water, purified on 58% Renografin-76 (Bristol-Myers Squibb, Princeton, N.J.) in water, as previously described (Palucka et al., *Nature Medicine*, 5:868-870 (1999)), and washed once more with water. The spores are then sedimented to a pellet at 10,000 x g and resuspended in 1% phenol in water. This yield of this process has been published to range from 0.5 x 10<sup>9</sup> to 5.0 x 10<sup>9</sup> spores per plate.

[0304] *Protection of mice against lethal spore challenge.* The LD<sub>50</sub> value of heat-shocked Sterne strain spores given by intramuscular (IM) injection is determined over a dose range of 10<sup>3</sup> to 10<sup>8</sup> spores. To evaluate protection in vaccinated mice against inhalation anthrax, challenge experiments are also performed by intratracheal (IT) spore administration, as described previously (Brook et al., *J. Med. Microbiol.*, 50:702-11 (2001)). Briefly, the tongue of immobilized and anesthetized mice are gently pulled outward and laterally with forceps, and the vaccine is delivered using a syringe fitted with a blunt 1.5 inch 22-gauge needle bent at a gentle angle, approximately 1 inch from the tip. We anticipate that the Sterne strain LD<sub>50</sub> value administered by IM or IT routes is approximately 10<sup>3</sup> in A/J mice, and up to 10-fold higher in Swiss Webster mice. The protection studies include up to 100 LD<sub>50</sub> dose spore challenge. All mice treated with spores are monitored daily, and experiments are terminated after 2 weeks and all protected mice are sacrificed. In all challenge experiments, the mean time to death is determined in non-surviving cohorts.

## EXAMPLE 45

Protective immunity of the *Listeria* vaccines against challenge with Vaccinia expressing OVA model antigen in mice

[0305] The vaccines of the invention show protective immunization against a *Listeria* challenge. To further illustrate the ability to immunize and protect against a pathogen, *Listeria* vaccines with or without OVA antigen and with and without S-59 UVA treatment (second method of Example 13, above) are used to immunize against another microbe, e.g. vaccinia virus that expresses the OVA antigen (VV-OVA). This will demonstrate that an antigen specific immunization against other microbes can be achieved with a *Listeria* vaccine.

[0306] Vaccinia virus (WR strain) expressing OVA is obtained from La Jolla Research Institute and prepared in Vero cells using Opticell chambers (BioCrystal, OH). Opticell chambers are seeded with Vero cells in Eagle's Minimal Essential Medium supplemented with L-glutamine, P/S (penicillin/streptomycin), NEAA (non-essential amino acids), NaHCO<sub>3</sub>, and 10% FBS. When the cells are at approximately 75% confluence, the growth medium is removed and replaced with fresh medium containing approximately  $1 \times 10^5$  PFU/mL of VV-OVA. When the monolayers show >50% cytopathic effect, cells and supernatants are harvested, subjected to three freeze-thaw cycles, clarified and stored at -80 °C. The titer is determined by a plaque assay on Vero-76 cells. Ovalbumin expression by the vaccinia is confirmed by a western blot analysis prior to injection into the mice.

[0307] C57Bl/6 mice (3 per group) are vaccinated IV according to Table 27 and challenged on day 7 with IP injection of  $1 \times 10^7$  PFU of the VV-OVA. On day 12, the mice are euthanized and the ovaries are harvested and observed for gross pathology. The ovaries are also assayed for vaccinia plaque forming units. Paired ovaries from individual mice are homogenized in 1 mL of buffer, frozen (liquid nitrogen) and thawed (37 °C) three cycles, with vortexing between each cycle, then stored at -80 °C. To assay, samples are thawed, centrifuged at 4 °C to remove debris, and serially diluted for application to Vero cells. Vero-76 cells are plated in 6-well tissue culture plates. When the cell monolayers reach a confluence of about 70-85%, the medium is aspirated from each well and the cells are inoculated with 1 mL of the appropriate dilution of ovary homogenate preparation. After at least one hour, the medium is aspirated and replaced with 3 mL of 1:1 2x growth medium:1.5% agarose. Plaques are enumerated after 3-4 days of culture.

Table 27 Vaccination of mice with *Listeria monocytogenes* expressing OVA and challenged with vaccinia expressing OVA.

Vaccine strain	Treatment	Vaccination days	Vaccination dose*
HBSS	-	0	100 $\mu$ L
$\Delta actA$	-	0	$1 \times 10^7$
$\Delta actA$	S-59 UVA	0, 1, 2	$1 \times 10^8$
$\Delta actA$ OVA	-	0	$1 \times 10^7$
$\Delta actA$ OVA	S-59 UVA	0, 1, 2	$1 \times 10^8$
$\Delta actA \Delta uvrAB$	-		$1 \times 10^7$
$\Delta actA \Delta uvrAB$	S-59 UVA	0, 1, 2	$1 \times 10^8$
$\Delta actA \Delta uvrAB$ OVA	-		$1 \times 10^7$
$\Delta actA \Delta uvrAB$ OVA	S-59 UVA	0, 1, 2	$1 \times 10^8$
$\Delta actA \Delta uvrAB$ OVA	Heat-killed	0, 1, 2	$1 \times 10^9$

\*Initial dose, day 1 and 2 doses are 10-fold lower. All doses 100  $\mu$ L HBSS.

[0308] All publications, patents, patent applications, and accession numbers (including both polynucleotide and polypeptide sequences) cited herein are hereby incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent or patent application were specifically and individually indicated to be so incorporated by reference.

Applicant's or agent's file reference 282172002840	International application No. PCT/US2004/003671
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>84</u> , line <u>32-33 (paragraph [0178])</u> as <u>DP-L402 <i>SuvrAB</i></u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input checked="" type="checkbox"/></span>	
Name of depositary institution <p align="center">American Type Culture Collection (ATCC)</p>	
Address of depositary institution (including postal code and country) <p align="center">10801 University Blvd. Manassas, Virginia 20110-2209 United States of America</p>	
Date of deposit <p align="center">03 October 2003</p>	Accession Number <p align="center">PTA-5563</p>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input checked="" type="checkbox"/></span>	
<p align="center"><i>Listeria monocytogenes</i> <math>\Delta actA \Delta suvrAB</math> mutant</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF  
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE****INTERNATIONAL FORM****RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3  
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.****To: (Name and Address of Depositor or Attorney)**Cerus Corporation  
Attn: John Tessman  
2411 Stanwell Drive  
Concord, CA 94520**Deposited on Behalf of:** Cerus Corporation**Identification Reference by Depositor:**Listeria monocytogenes actA/inIB double mutant: actA/inIB  
Listeria monocytogenes actA/uvrAB double mutant: actA/uvrAB**Patent Deposit Designation**PTA-5562  
PTA-5563

The deposits were accompanied by:    a scientific description, a proposed taxonomic description indicated above. The deposits were received October 3, 2003 by this International Depository Authority and have been accepted.

**AT YOUR REQUEST:**   X   We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

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The viability of the cultures cited above was tested October 15, 2003. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

**Signature of person having authority to represent ATCC:**  
Marie Harris, Patent Specialist, ATCC Patent Depository**Date:** November 10, 2003cc: Alicia Hager  
(Ref: Docket or Case No.: 28217-3002900)

Applicant's or agent's file reference 282172002840	International application No. PCT/US2004/00367 1
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**INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL**

(PCT Rule 13bis)

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A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>100</u> , line <u>25-26 (paragraph [0208])</u> as <u>DP-L4406actA</u>	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution <p align="center">American Type Culture Collection (ATCC)</p>	
Address of depositary institution (including postal code and country) <p>10801 University Blvd. Manassas, Virginia 20110-2209 United States of America</p>	
Date of deposit <p align="center">03 October 2003</p>	Accession Number <p align="center">PTA-5562</p>
C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input checked="" type="checkbox"/></span>	
<p align="center"><i>Listeria monocytogenes</i> <math>\Delta actA \Delta inlB</math> double mutant</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
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**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF  
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE****INTERNATIONAL FORM****RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3  
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.****To: (Name and Address of Depositor or Attorney)**Cerus Corporation  
Attn: John Tessman  
2411 Stanwell Drive  
Concord, CA 94520**Deposited on Behalf of:** Cerus Corporation**Identification Reference by Depositor:****Patent Deposit Designation**Listeria monocytogenes actA/inlB double mutant: actA/inlB  
Listeria monocytogenes actA/uvrAB double mutant: actA/uvrABPTA-5562  
PTA-5563

The deposits were accompanied by:   a scientific description  , a proposed taxonomic description indicated above. The deposits were received October 3, 2003 by this International Depository Authority and have been accepted.

**AT YOUR REQUEST:**   X   We will inform you of requests for the strains for 30 years.

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International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

Marie Harris  
Marie Harris, Patent Specialist, ATCC Patent Depository

Date: November 10, 2003

cc: Alicia Hager  
(Ref: Docket or Case No.: 28217-3002900)

## CLAIMS

What we claim is:

1. A vaccine comprising a free-living microbe, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation.
2. The vaccine of claim 1, wherein the nucleic-acid targeted compound is a nucleic acid alkylator.
3. The vaccine of claim 2, wherein the nucleic acid alkylator is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester.
4. The vaccine of claim 1, wherein the nucleic acid targeted compound is activated by irradiation.
5. The vaccine of claim 4, wherein the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation.
6. The vaccine of claim 5, wherein the nucleic acid targeted compound is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen.
7. The vaccine of claim 1, wherein the microbe comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified.
8. The vaccine of claim 7, wherein the microbe is defective with respect to a DNA repair enzyme.
9. The vaccine of claim 8, wherein the genetic mutation is in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*.

10. The vaccine of claim 9, wherein the microbe comprises genetic mutations in both *uvrA* and *uvrB*, or in functional equivalents of both *uvrA* and *uvrB*.
11. The vaccine of claim 8, which is defective with respect to RecA, or the functional equivalent of RecA.
12. The vaccine of claim 1, wherein the microbe is a bacterium.
13. The vaccine of claim 12, wherein the microbe is *Mycobacterium tuberculosis*.
14. The vaccine of claim 12, wherein the microbe is *Bacillus anthracis*.
15. The vaccine of claim 12, wherein the microbe is *Listeria monocytogenes*.
16. The vaccine of claim 15, wherein the microbe comprises at least one mutation in both *uvrA* and *uvrB*.
17. The vaccine of claim 16, wherein the microbe further comprises a mutation in the *actA* gene, the *inlB* gene, or both genes.
18. The vaccine of claim 1, wherein the microbe comprises a heterologous nucleic acid sequence encoding an antigen.
19. The vaccine of claim 1, wherein the vaccine further comprises a pharmaceutically acceptable carrier or an adjuvant.
20. A method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the vaccine of claim 1.
21. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the vaccine of claim 1, wherein the microbe expresses the antigen.

22. An isolated professional antigen-presenting cell comprising a free-living microbe, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation.
23. The professional antigen-presenting cell of claim 22, which is a dendritic cell.
24. The professional antigen-presenting cell of claim 22, wherein the nucleic-acid targeted compound is a nucleic acid alkylator.
25. The professional antigen-presenting cell of claim 24, wherein the nucleic acid alkylator is  $\beta$ -alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester.
26. The professional antigen-presenting cell of claim 22, wherein the nucleic acid targeted compound is activated by irradiation.
27. The professional antigen-presenting cell of claim 26, wherein the nucleic acid targeted compound is a psoralen compound activated by UVA irradiation.
28. The professional antigen-presenting cell of claim 27, wherein the nucleic acid targeted compound is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen.
29. The professional antigen-presenting cell of claim 22, wherein the microbe comprises a genetic mutation that attenuates the ability of the microbe to repair its nucleic acid that has been modified.
30. The professional antigen-presenting cell of claim 29, wherein the microbe is defective with respect to a DNA repair enzyme.
31. The professional antigen-presenting cell of claim 30, wherein the genetic mutation is in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*.

32. The professional antigen-presenting cell of claim 31, wherein the microbe comprises genetic mutations in both *uvrA* and *uvrB*, or in functional equivalents of both *uvrA* and *uvrB*.
33. The professional antigen-presenting cell of claim 31, wherein the microbe is defective with respect to RecA, or a functional equivalent of Rec A.
34. The professional antigen-presenting cell of claim 23, wherein the microbe is a bacterium.
35. The professional antigen-presenting cell of claim 34, wherein the microbe is *Mycobacterium tuberculosis*.
36. The professional antigen-presenting cell of claim 34, wherein the microbe is *Listeria monocytogenes*.
37. The professional antigen-presenting cell of claim 32, wherein the microbe comprises at least one mutation in both *uvrA* and *uvrB*.
38. The professional antigen-presenting cell of claim 22, wherein the microbe comprises a heterologous nucleic acid sequence encoding an antigen.
39. A vaccine comprising the professional antigen-presenting cell of claim 22.
40. A method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the professional antigen-presenting cell of claim 22.
41. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the professional antigen-presenting cell of claim 22, wherein the microbe comprises a nucleic acid sequence encoding the antigen.

42. A method of activating naïve T cells *in vitro*, comprising contacting the naïve T cells with the professional antigen-presenting cell of claim 22 under suitable conditions and for a sufficient time to activate the naïve T cells.
43. A method of loading professional antigen-presenting cells with an antigen comprising contacting the professional antigen-presenting cells *in vitro* with a free-living microbe that comprises a nucleic acid sequence encoding the antigen, under suitable conditions and for a sufficient time to load the professional antigen-presenting cells, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation.
44. A method of activating and/or maturing professional antigen-presenting cells comprising contacting the professional antigen-presenting cells *in vitro* with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, under suitable conditions and for a sufficient time to activate and/or to allow the maturation of the professional antigen-presenting cells, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation.
45. A method of preventing or treating a disease in a host, comprising the following steps. (a) loading professional antigen-presenting cells with an antigen by contacting the cells with a free-living microbe that comprises a nucleic acid sequence encoding an antigen, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation; and (b) administering an effective amount of a composition comprising the loaded professional antigen-presenting cells to the host.
46. A method of inducing an immune response to an antigen in a host, comprising the following steps. (a) loading professional antigen-presenting cells with the antigen by contacting the cells with a free-living microbe that comprises a nucleic acid sequence encoding the antigen, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so



that the microbe is attenuated for proliferation; and (b) administering an effective amount of a composition comprising the loaded professional antigen-presenting cells to the host.

47. An isolated mutant *Listeria monocytogenes* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid.

48. The mutant strain of claim 47, which is defective with respect to at least one DNA repair enzyme.

49. The mutant strain of claim 47 which is attenuated with respect to UvrA, UvrB, or both UvrA and UvrB.

50. The mutant strain of claim 49, which comprises a genetic mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* genes.

51. The mutant strain of claim 47, wherein the nucleic acid of the bacteria of the strain have been modified so that the bacteria are attenuated for proliferation.

52. The mutant strain of claim 47, which is selected from the group consisting of a *Listeria monocytogenes actA/uvrAB* strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5563, or a mutant of the deposited strain which is defective with respect to UvrA, UvrB, and ActA.

53. The mutant strain of claim 52, which is the *Listeria monocytogenes actA/inlB* strain deposited with the American Type Culture Collection (ATCC) and identified by accession number PTA-5562.

54. A vaccine comprising (a) the mutant strain of claim 47, and (b) a pharmaceutically acceptable carrier or adjuvant.

55. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of a composition comprising the strain of claim 47, wherein the strain comprises a nucleic acid molecule encoding the antigen.

56. A method of preventing or treating a disease in a host, comprising administering to the host an effective amount of a composition comprising the strain of claim 47.
57. A professional antigen-presenting cell comprising the strain of claim 47.
58. An isolated mutant *Bacillus anthracis* strain comprising a genetic mutation that attenuates its ability to repair its nucleic acid.
59. The mutant strain of claim 58, which is defective with respect to at least one DNA repair enzyme.
60. The mutant strain of claim 59 which is attenuated with respect to UvrA, UvrB, or both UvrA and UvrB.
61. The mutant strain of claim 60, which comprises a genetic mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* genes.
62. The mutant strain of claim 58, which comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain.
63. The mutant strain of claim 58, wherein the nucleic acid of the bacteria of the strain have been modified so that the bacteria are attenuated for proliferation.
64. A method of inducing an immune response in a host to a *Bacillus anthracis* antigen comprising administering to the host an effective amount of a composition comprising the mutant strain of claim 58.
65. A method of preventing or treating a *Bacillus anthracis* infection in a host, comprising administering to the host an effective amount of a composition comprising the mutant strain of claim 58.
66. A vaccine comprising a free-living microbe which is defective with respect to at least one DNA repair enzyme.

67. The vaccine of claim 66, wherein the microbe comprises a genetic mutation in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*.

68. The vaccine of claim 67, wherein the microbe comprises genetic mutations in both *uvrA* and *uvrB*, or in functional equivalents of both *uvrA* and *uvrB*.

69. The vaccine of claim 66, which is defective with respect to *RecA*, or the functional equivalent of *RecA*.

70. The vaccine of claim 66, wherein the microbe is a bacterium.

71. The vaccine of claim 66, wherein the microbe comprises a heterologous nucleic acid sequence encoding an antigen.

72. The vaccine of claim 66, wherein the vaccine further comprises a pharmaceutically acceptable carrier or an adjuvant.

73. A method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the vaccine of claim 66.

74. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the vaccine of claim 66, wherein the microbe expresses the antigen.

75. An isolated professional antigen-presenting cell comprising a free-living microbe which is defective with respect to at least one DNA repair enzyme.

76. The antigen-presenting cell of claim 75, wherein the microbe comprises a genetic mutation in one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*, or in a functional equivalent of one or more gene selected from the group consisting of *phrB*, *uvrA*, *uvrB*, *uvrC*, *uvrD* and *recA*.

77. The antigen-presenting cell of claim 76, wherein the microbe comprises genetic mutations in both *uvrA* and *uvrB*, or in functional equivalents of both *uvrA* and *uvrB*.
78. The antigen-presenting cell of claim 75, which is defective with respect to RecA, or the functional equivalent of RecA.
79. The antigen-presenting cell of claim 75, wherein the microbe is a bacterium.
80. The antigen-presenting cell of claim 75, wherein the microbe comprises a heterologous nucleic acid sequence encoding an antigen.
81. A method of preventing or treating a disease in a host, comprising administering to the host an effective amount of the antigen-presenting cell of claim 75.
82. A method of inducing an immune response in a host to an antigen comprising administering to the host an effective amount of the antigen-presenting cell of claim 75, wherein the microbe expresses the antigen.
83. A free-living microbe for medical use, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation.
84. An antigen-presenting cell for medical use, wherein the antigen-presenting cell comprises a free-living microbe, wherein the nucleic acid of the microbe has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the microbe is attenuated for proliferation.
85. A mutant *Listeria monocytogenes* strain for medical use, wherein the mutant *Listeria monocytogenes* strain comprises a genetic mutation that attenuates its ability to repair its nucleic acid.
86. A free-living microbe for medical use, wherein the microbe is defective with respect to at least one DNA repair enzyme.

87. The microbe of claim 86, which is *Bacillus anthracis* or *Listeria monocytogenes*.
88. An antigen-presenting cell for medical use, wherein the antigen-presenting cell comprises a free-living microbe, wherein the microbe is defective with respect to at least one DNA repair enzyme.
89. A kit comprising both (a) composition comprising the mutant *Listeria monocytogenes* strain of claim 47, the mutant *Bacillus anthracis* strain of claim 58, or a free-living microbe, wherein the nucleic acid of the free-living microbe has been modified by reaction with a nucleic acid targeted compound; and (b) instructions for the use of the composition in the prevention or treatment of a disease in a host.

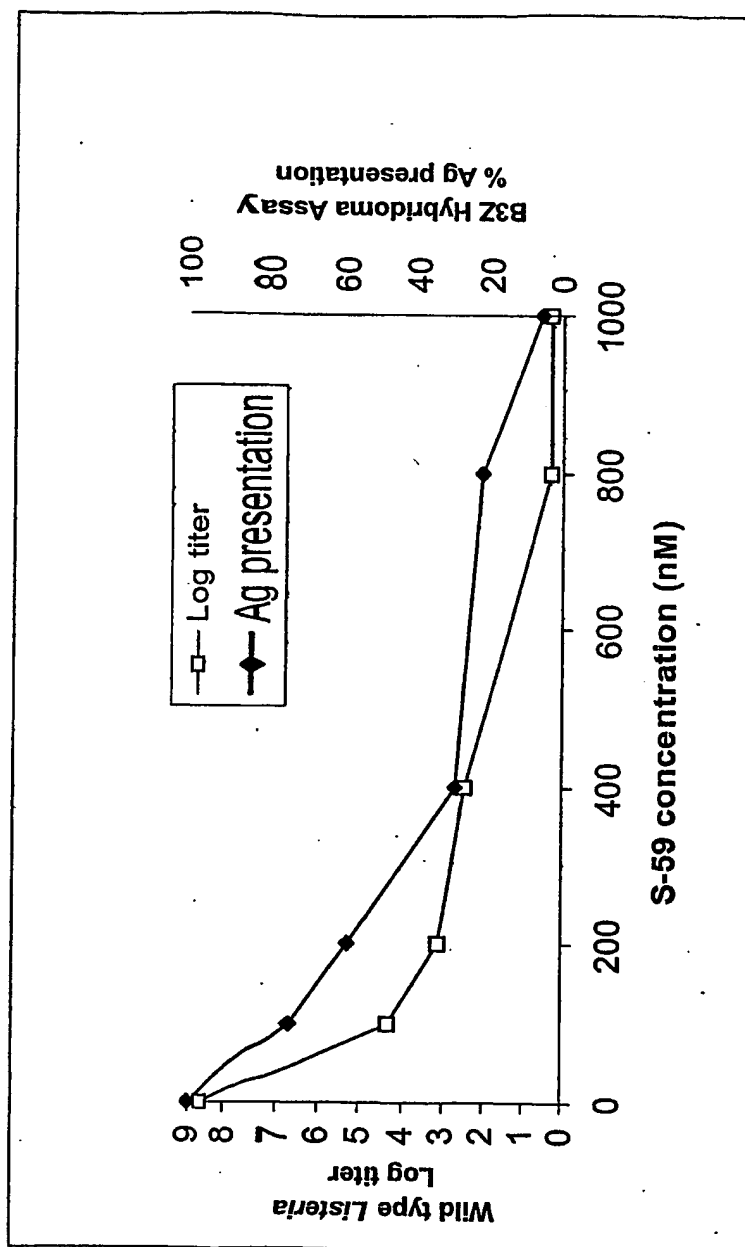


Figure 1

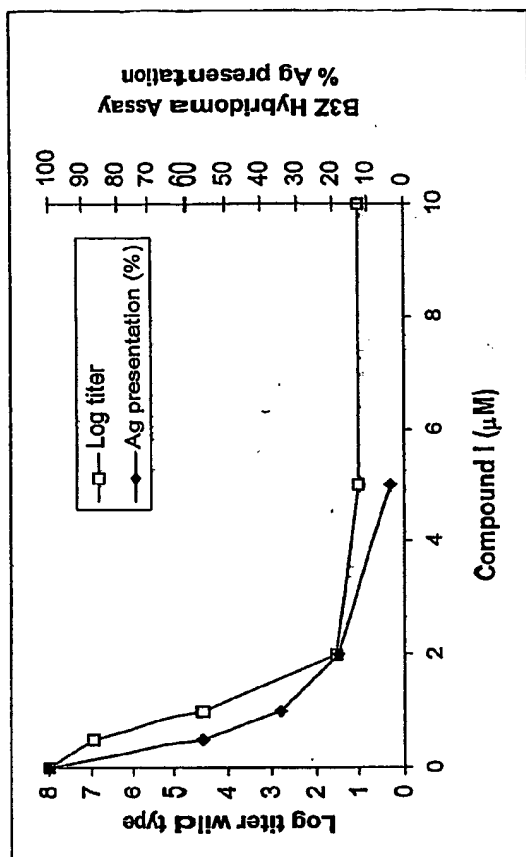


Figure 2A

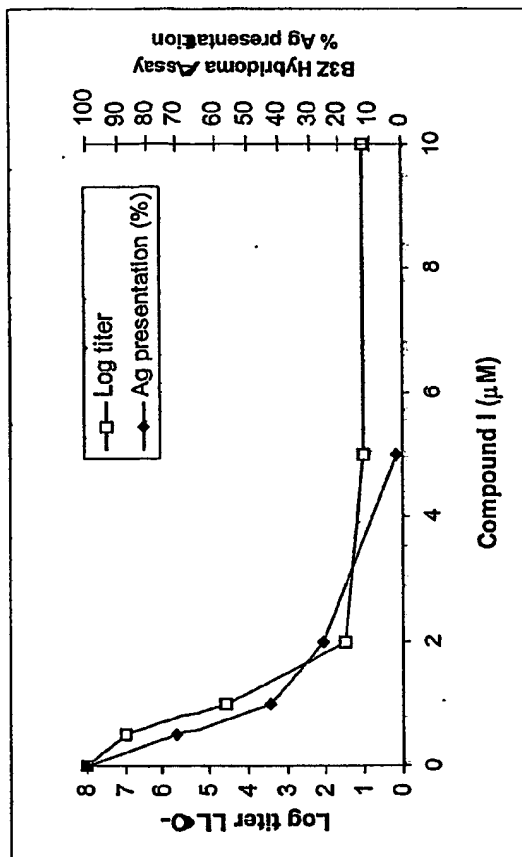


Figure 2B

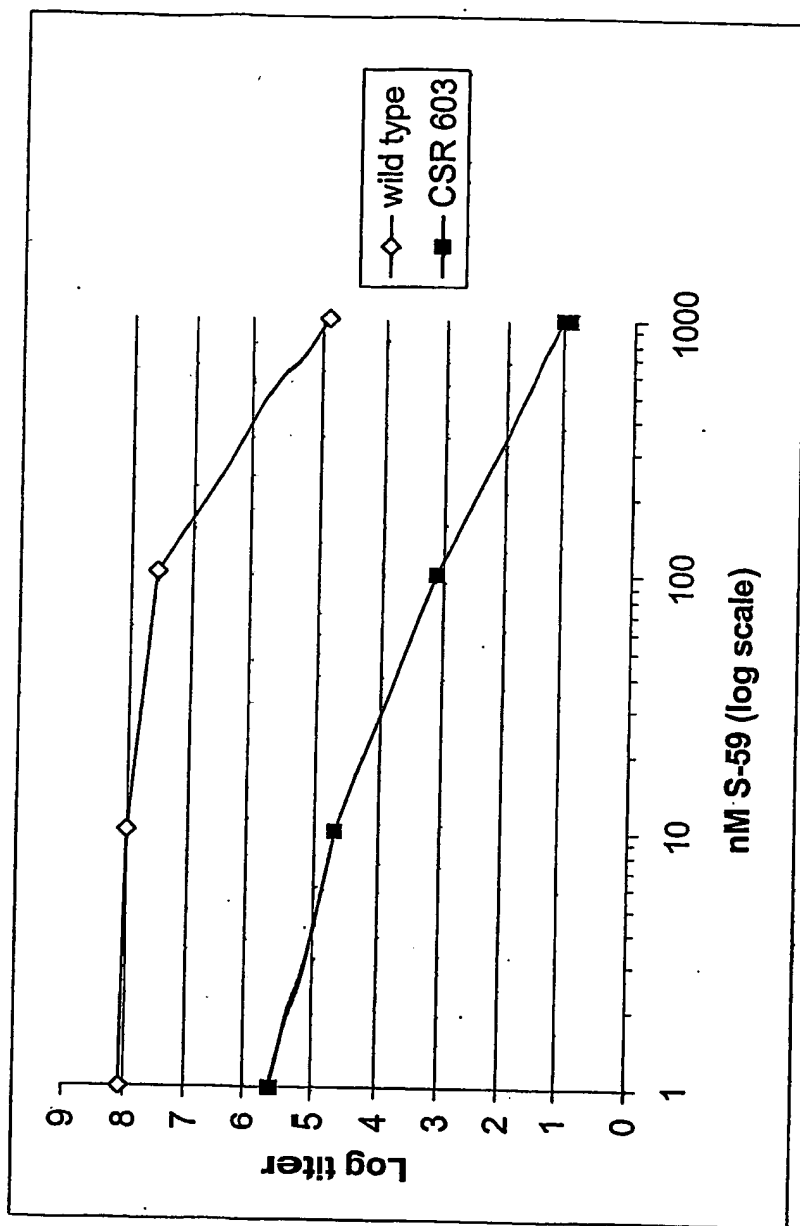


Figure 3



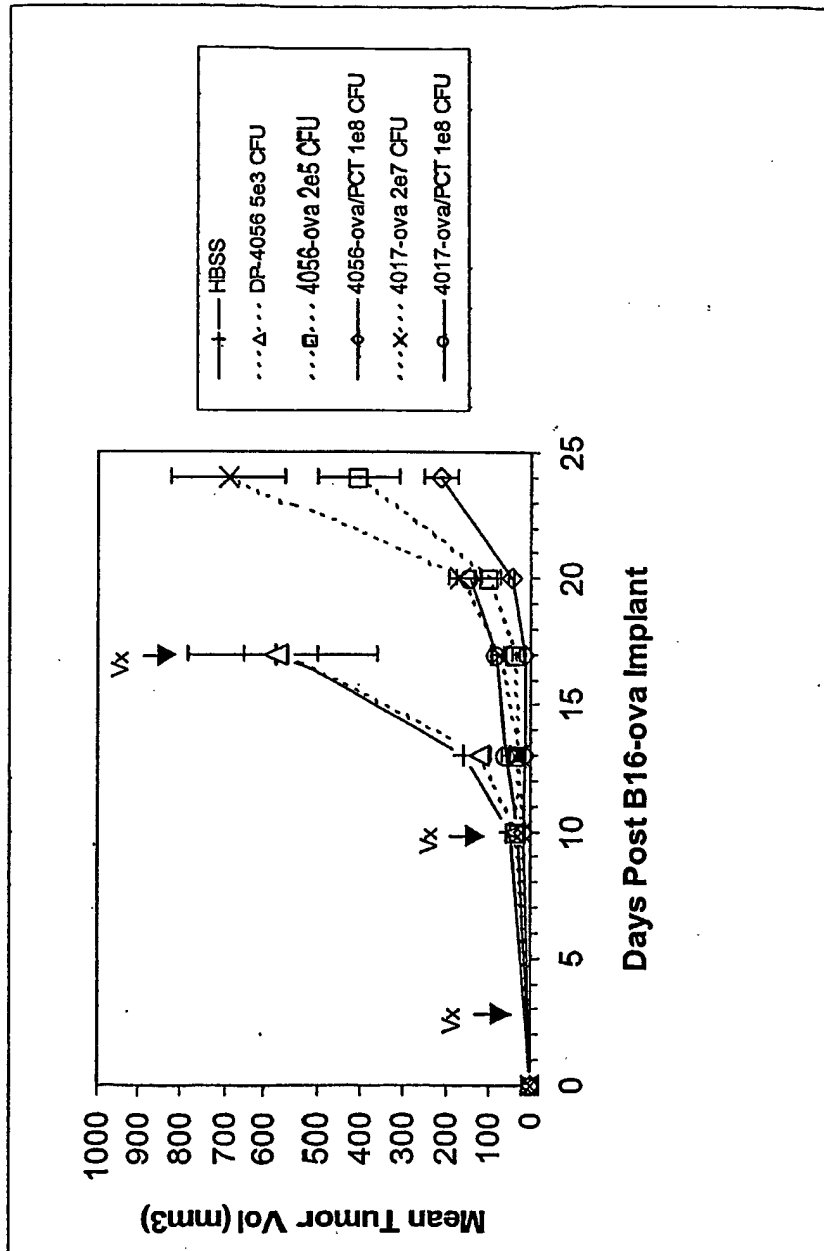


Figure 4

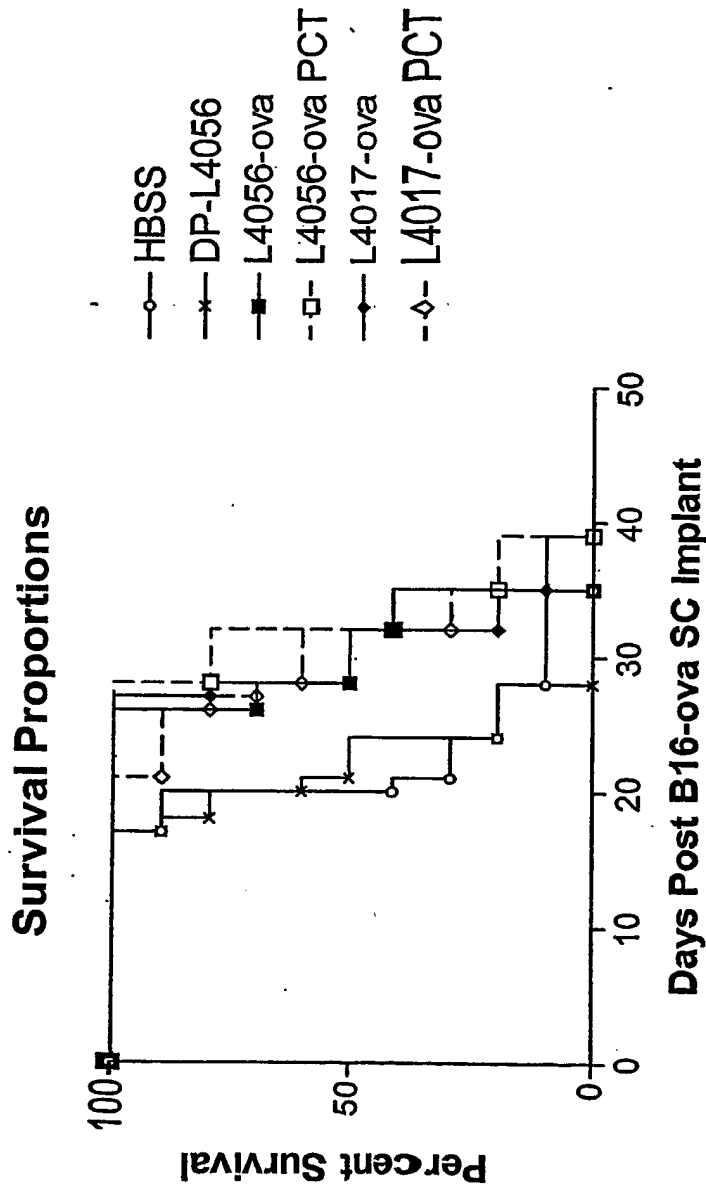
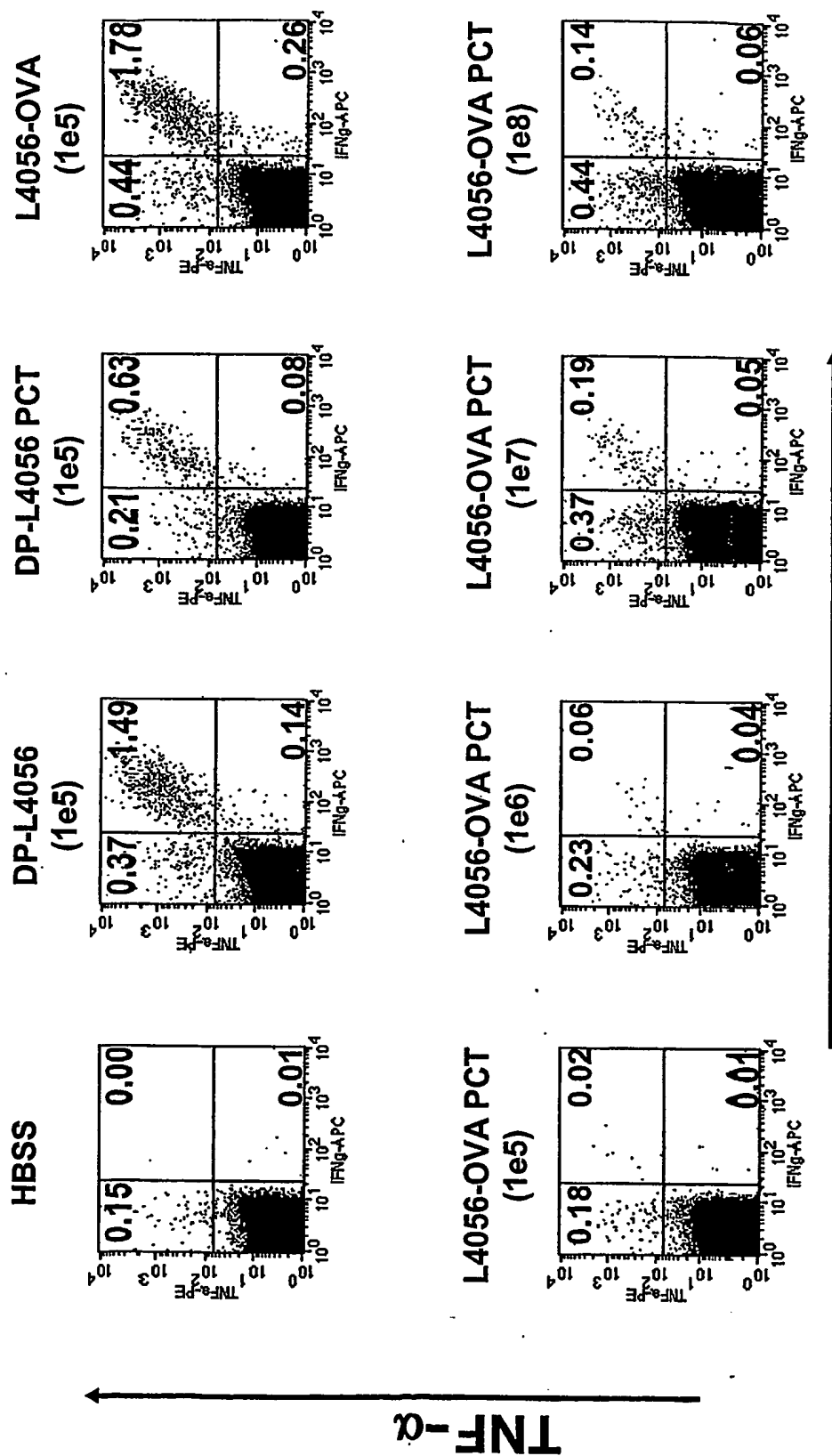


Figure 5

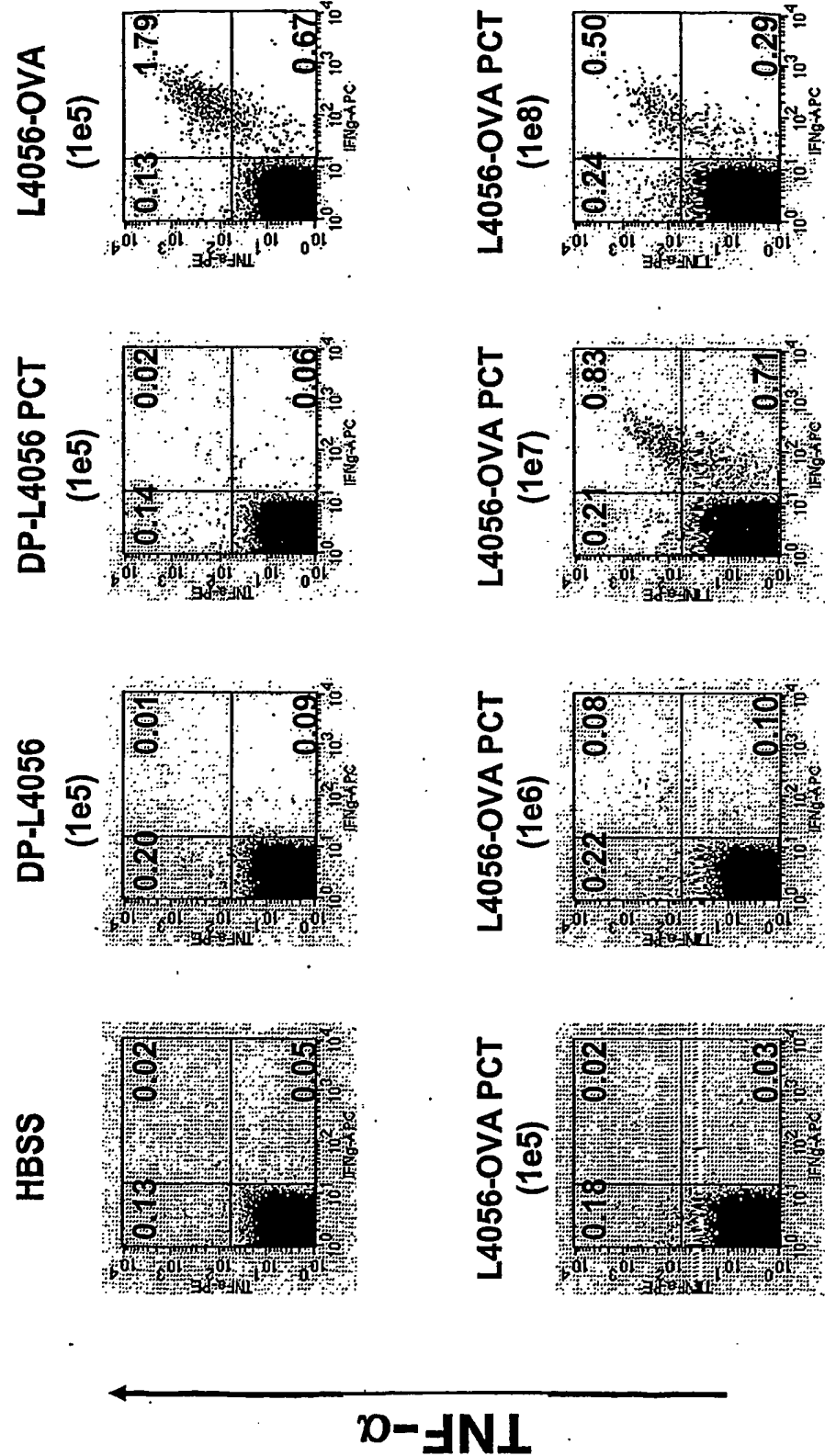
# LLO<sub>190-201</sub>-specific CD4<sup>+</sup> T cell response



IFN-γ

Figure 6A

OVA-specific CD8<sup>+</sup> T cell response



IFN- $\gamma$

Figure 6B

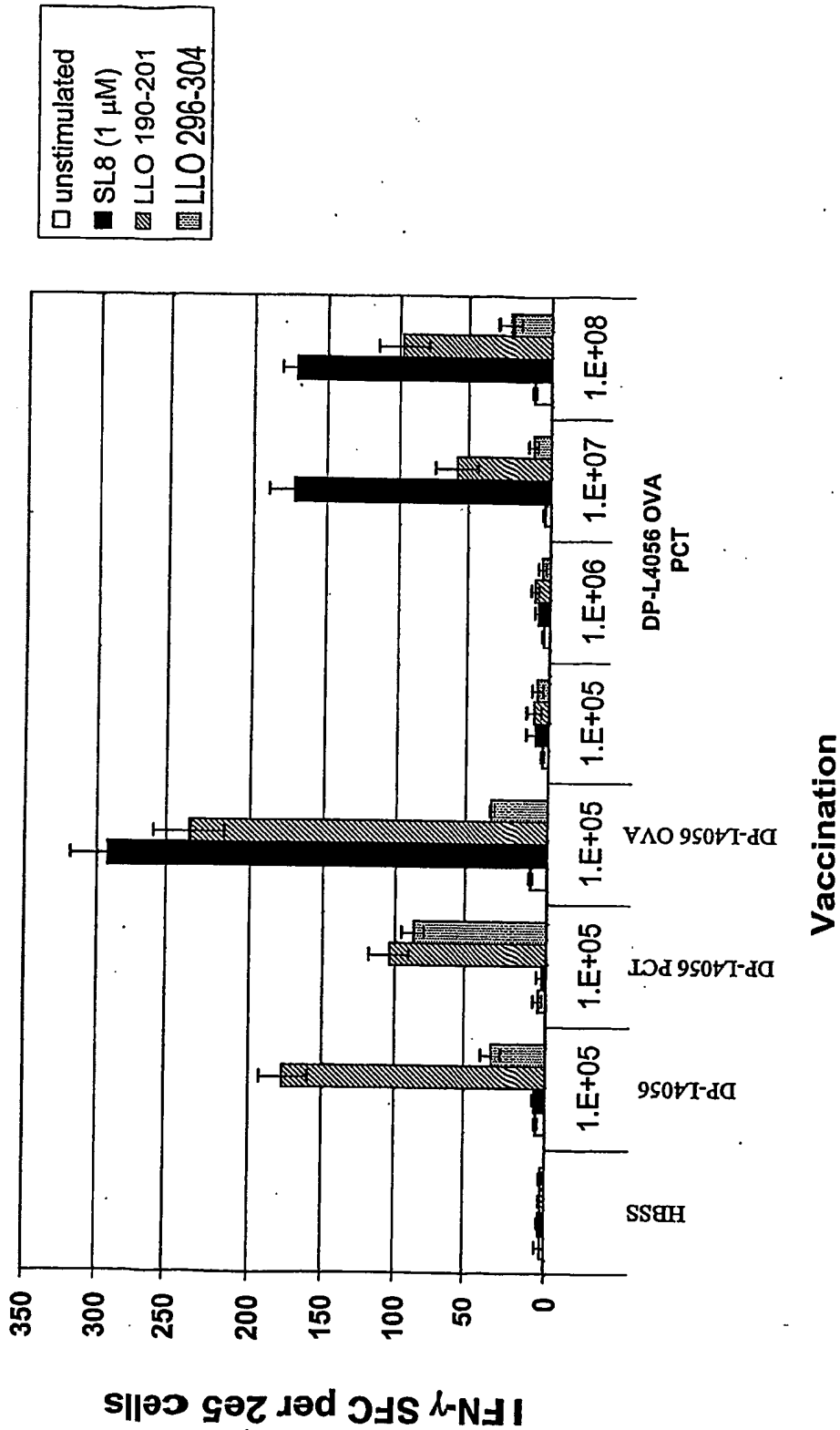


Figure 7

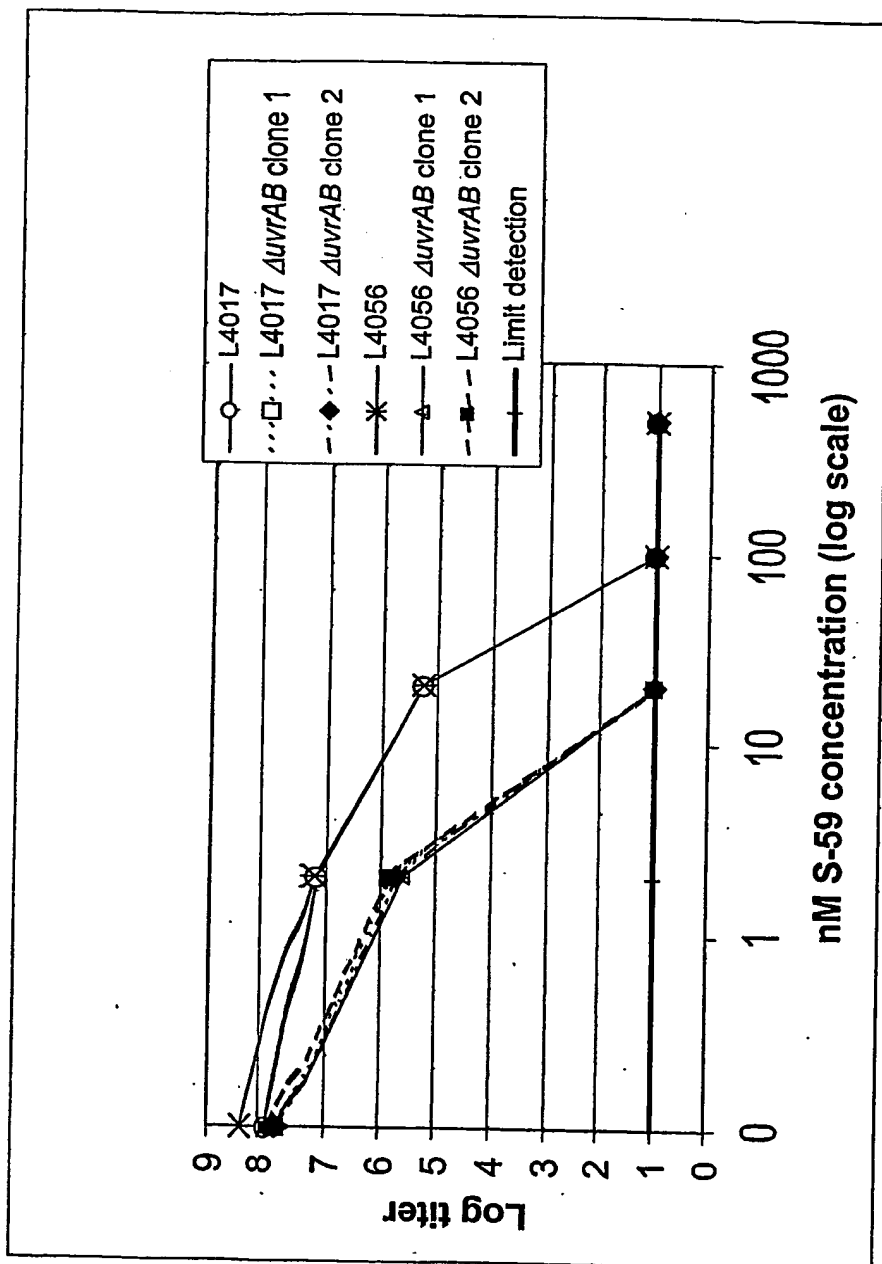


Figure 8A

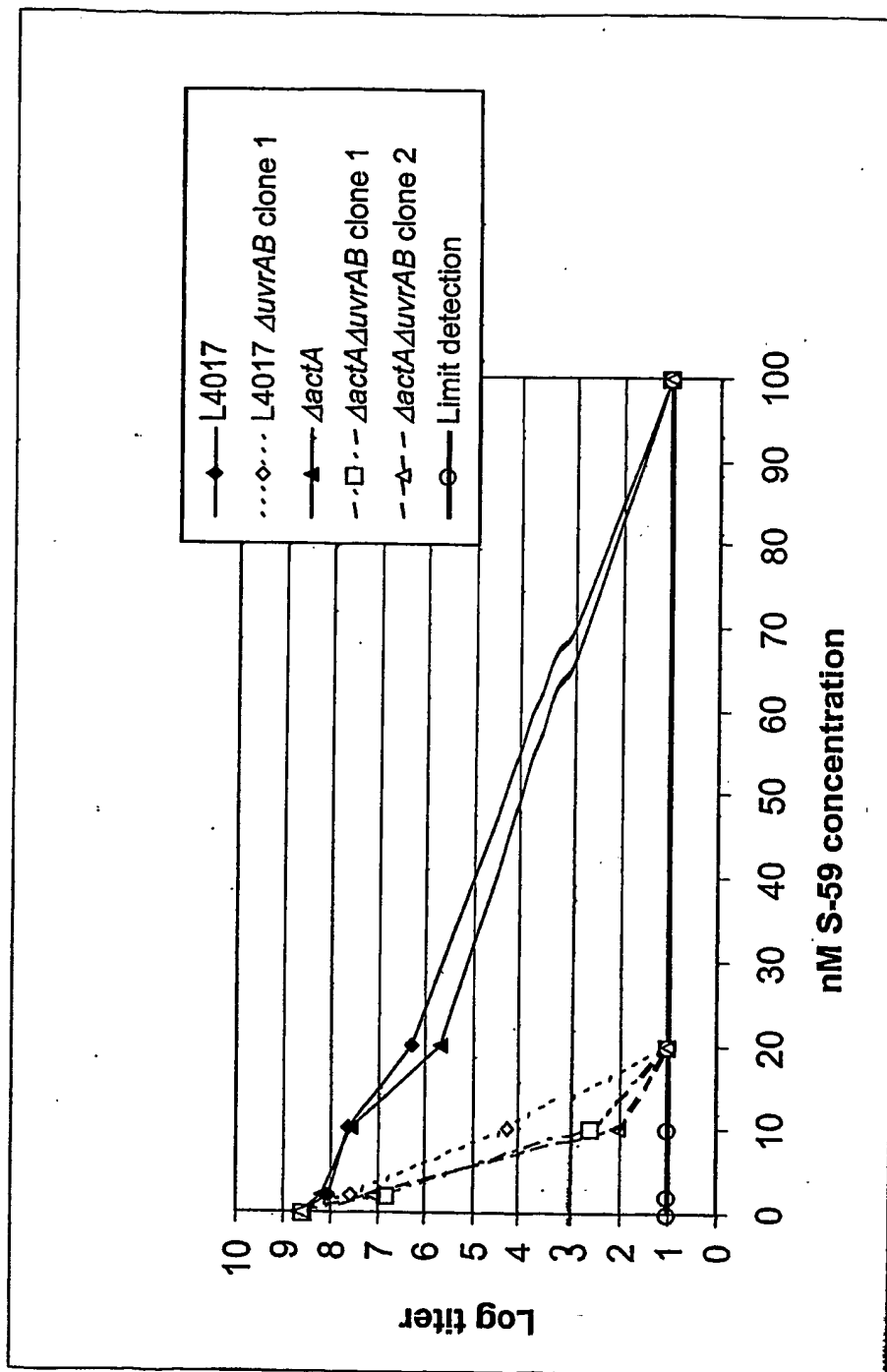


Figure 8B

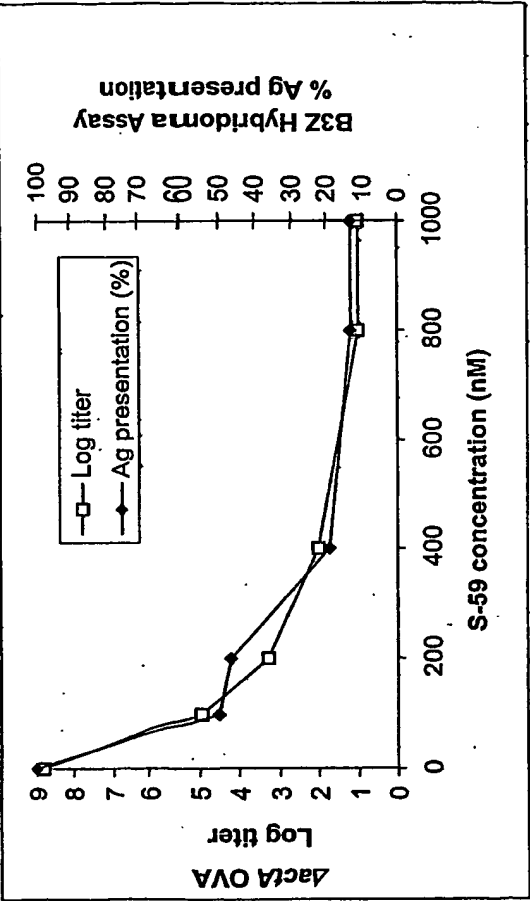


Figure 9A

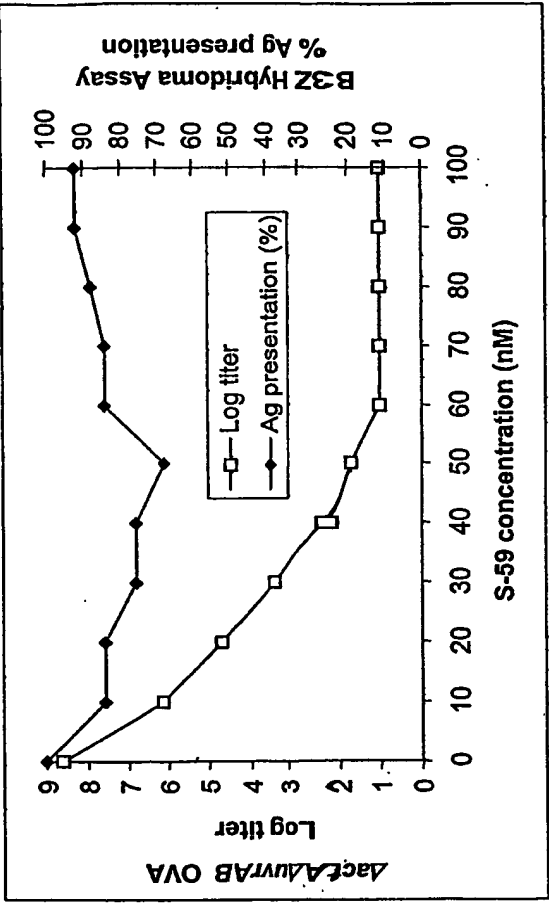


Figure 9B



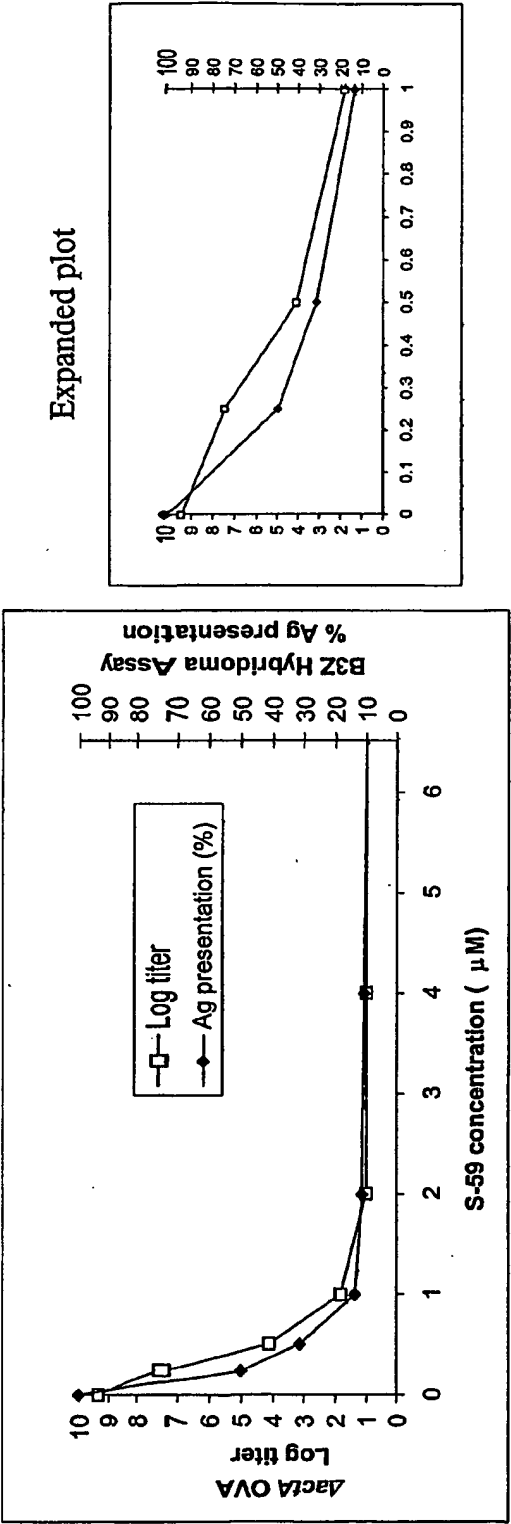


Figure 9C

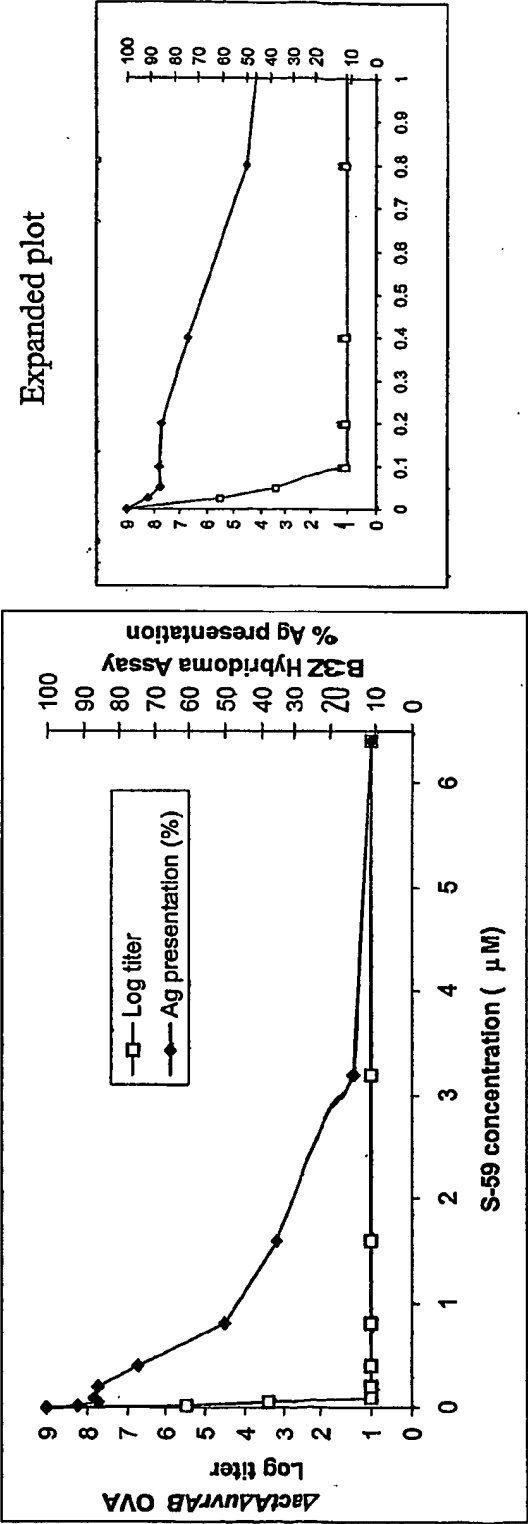


Figure 9D

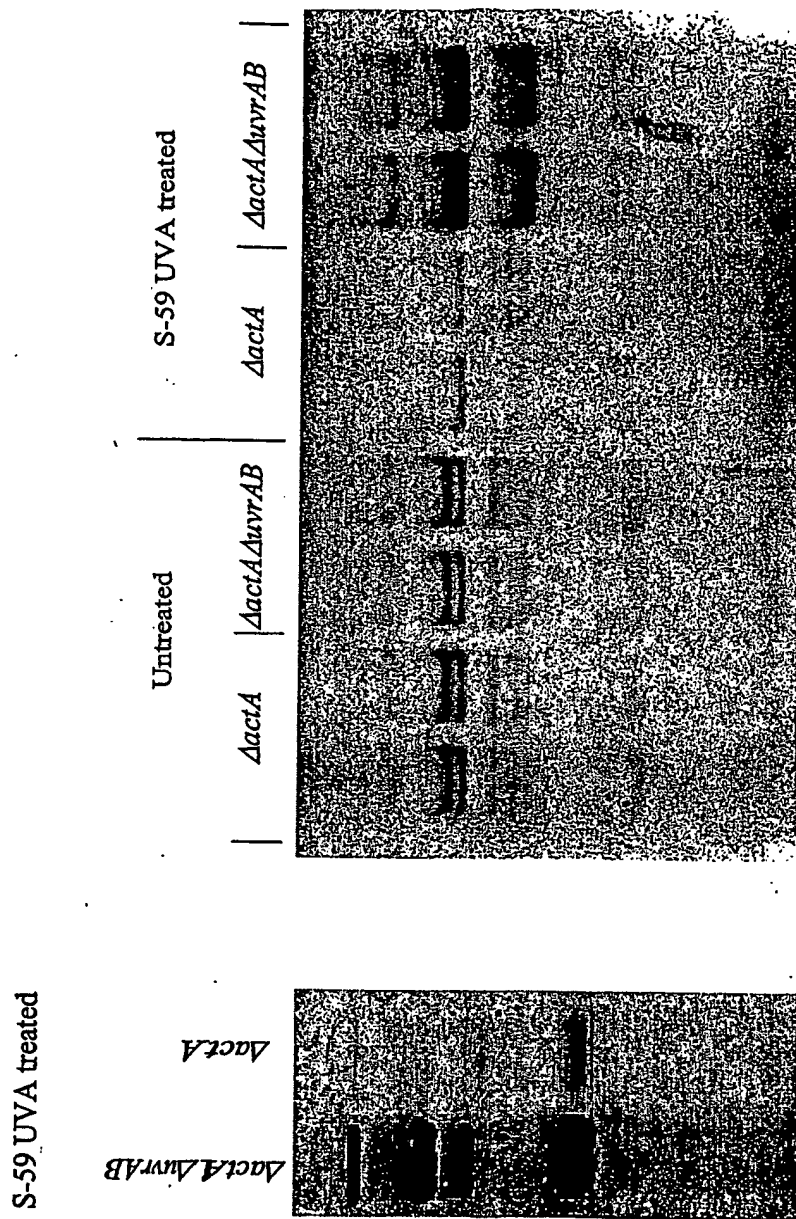


Figure 10

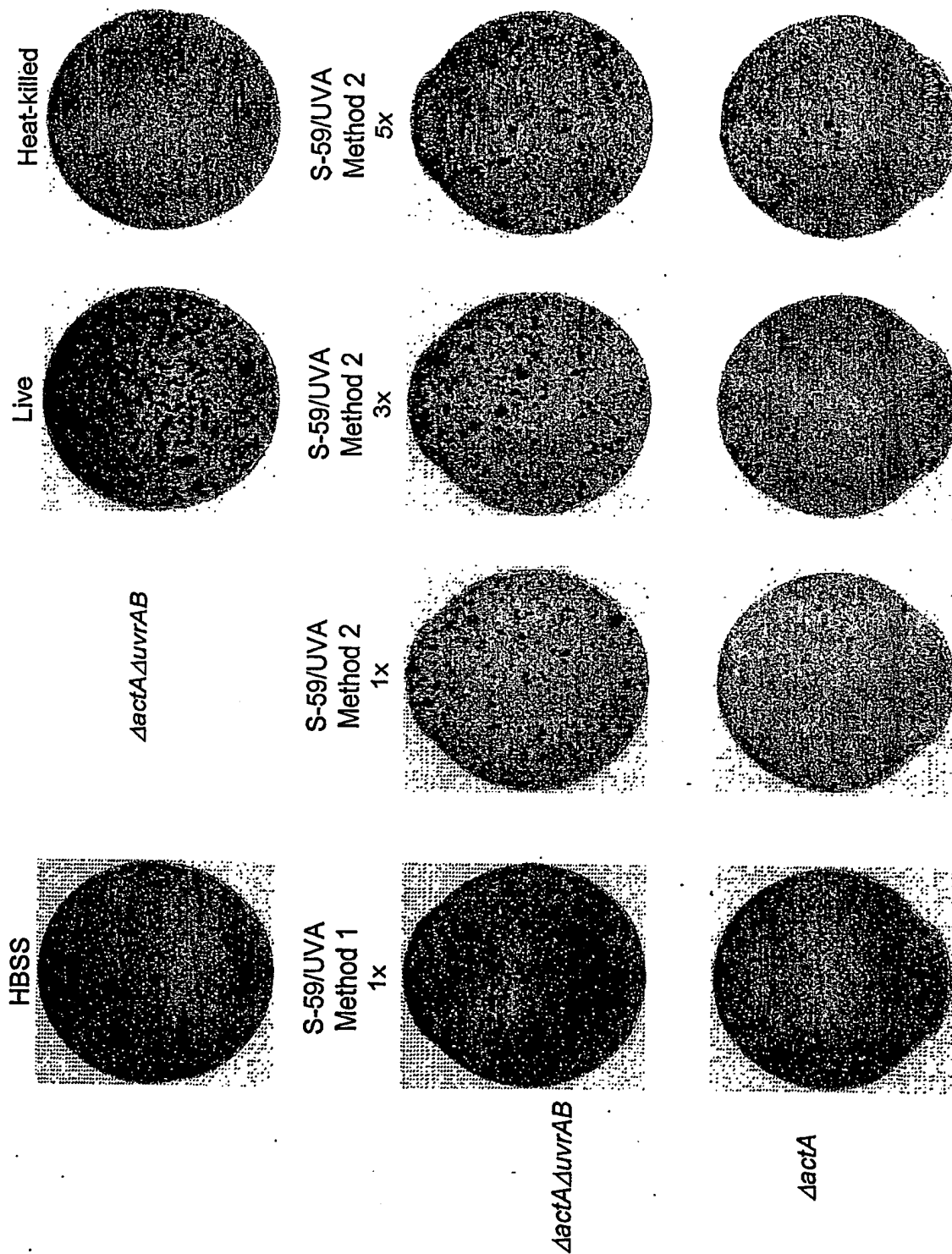


Figure 11A

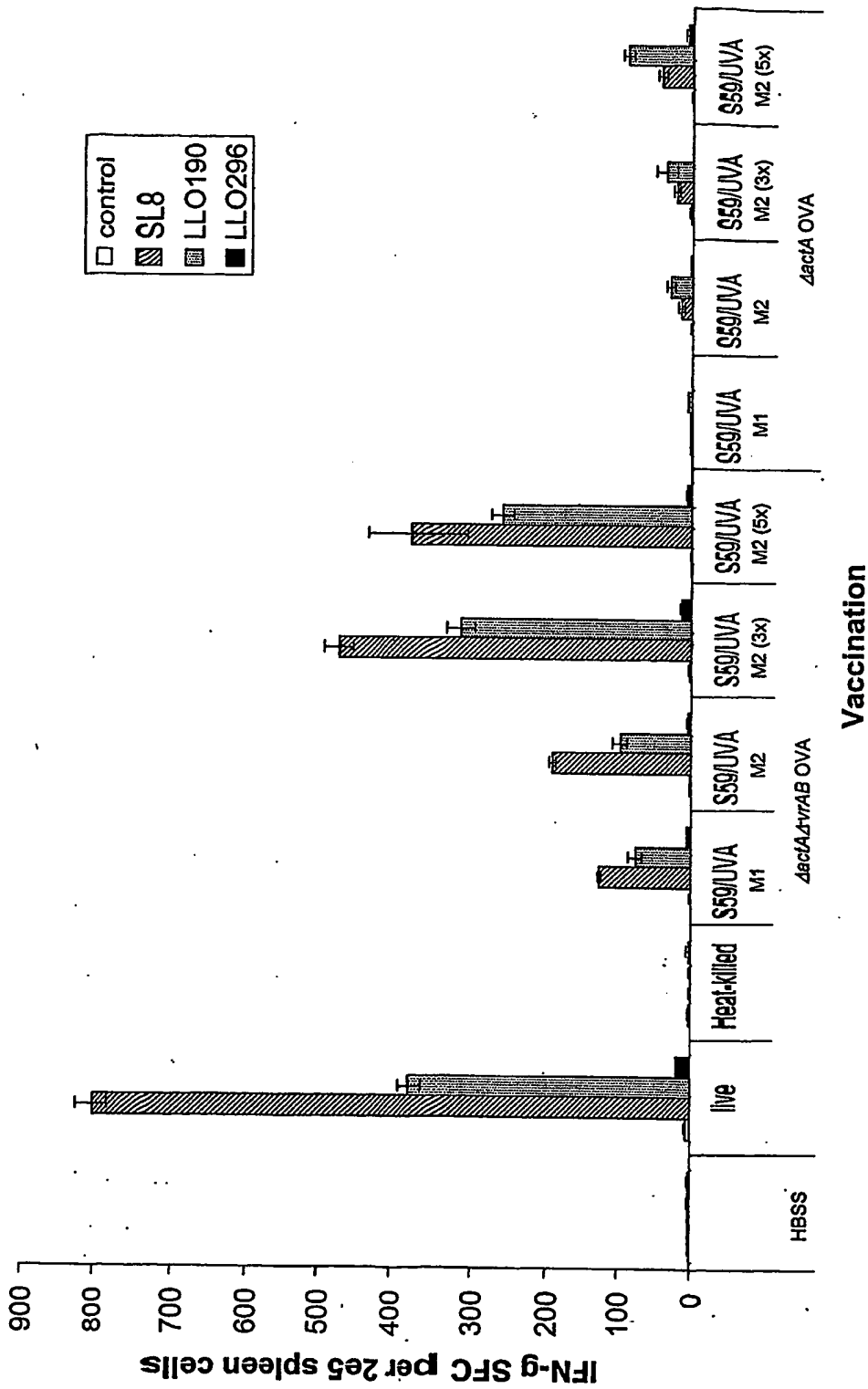


Figure 11B

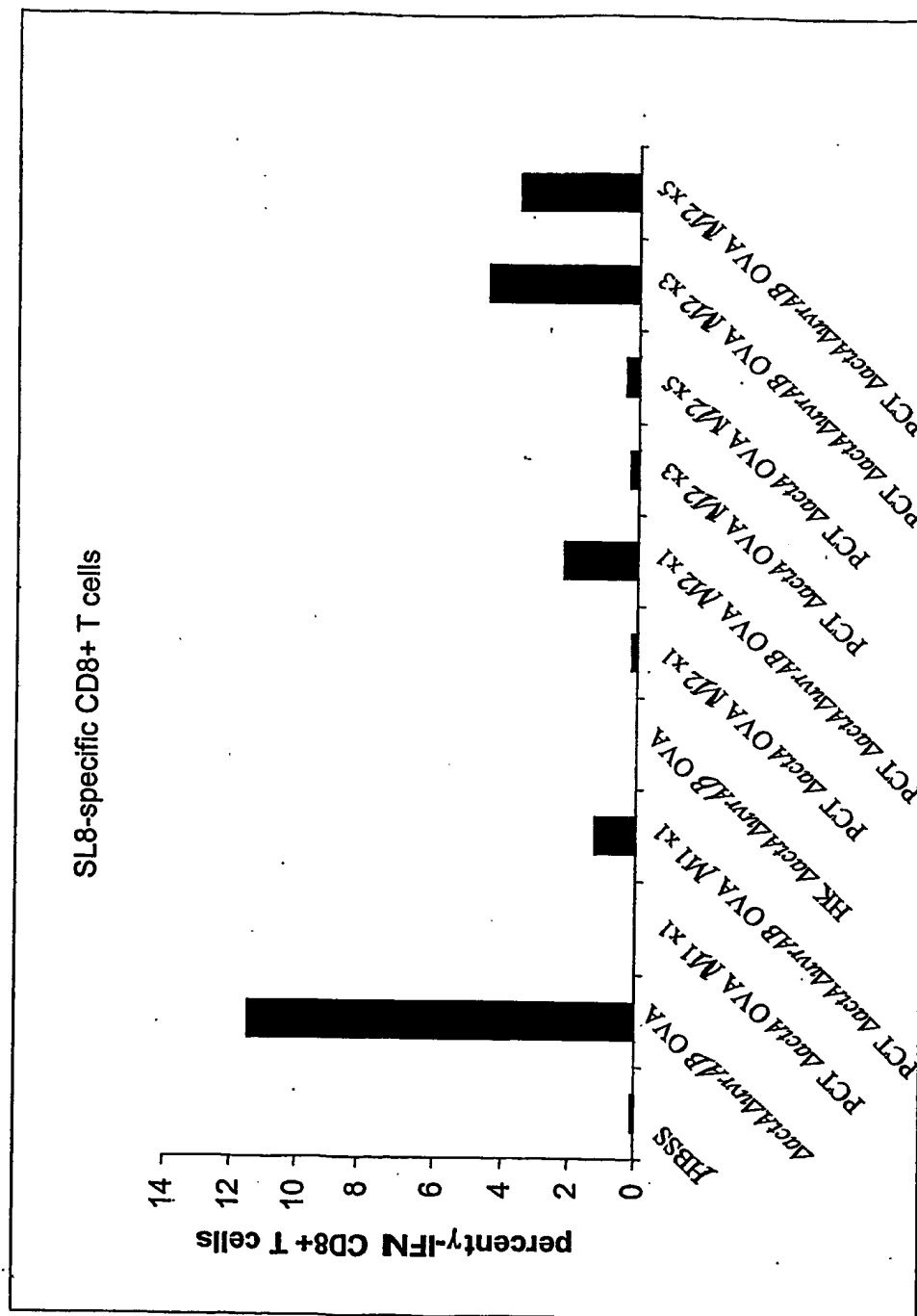


Figure 12A

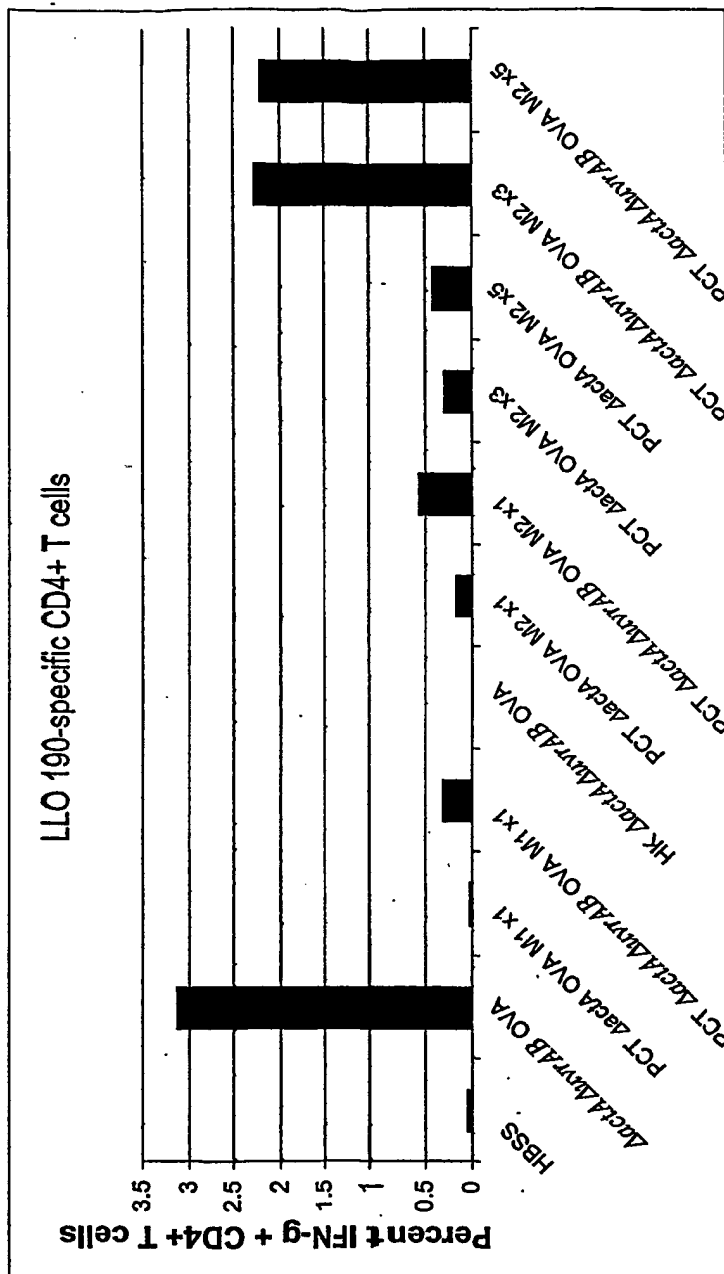


Figure 12B

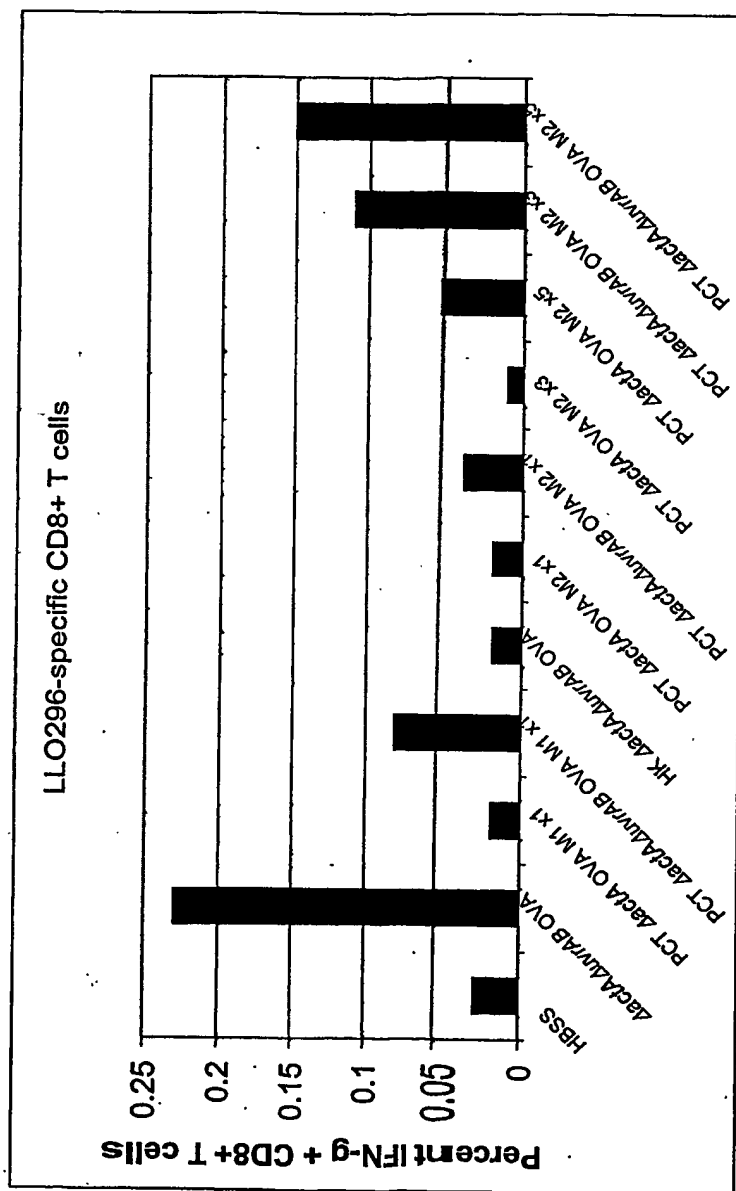


Figure 12C

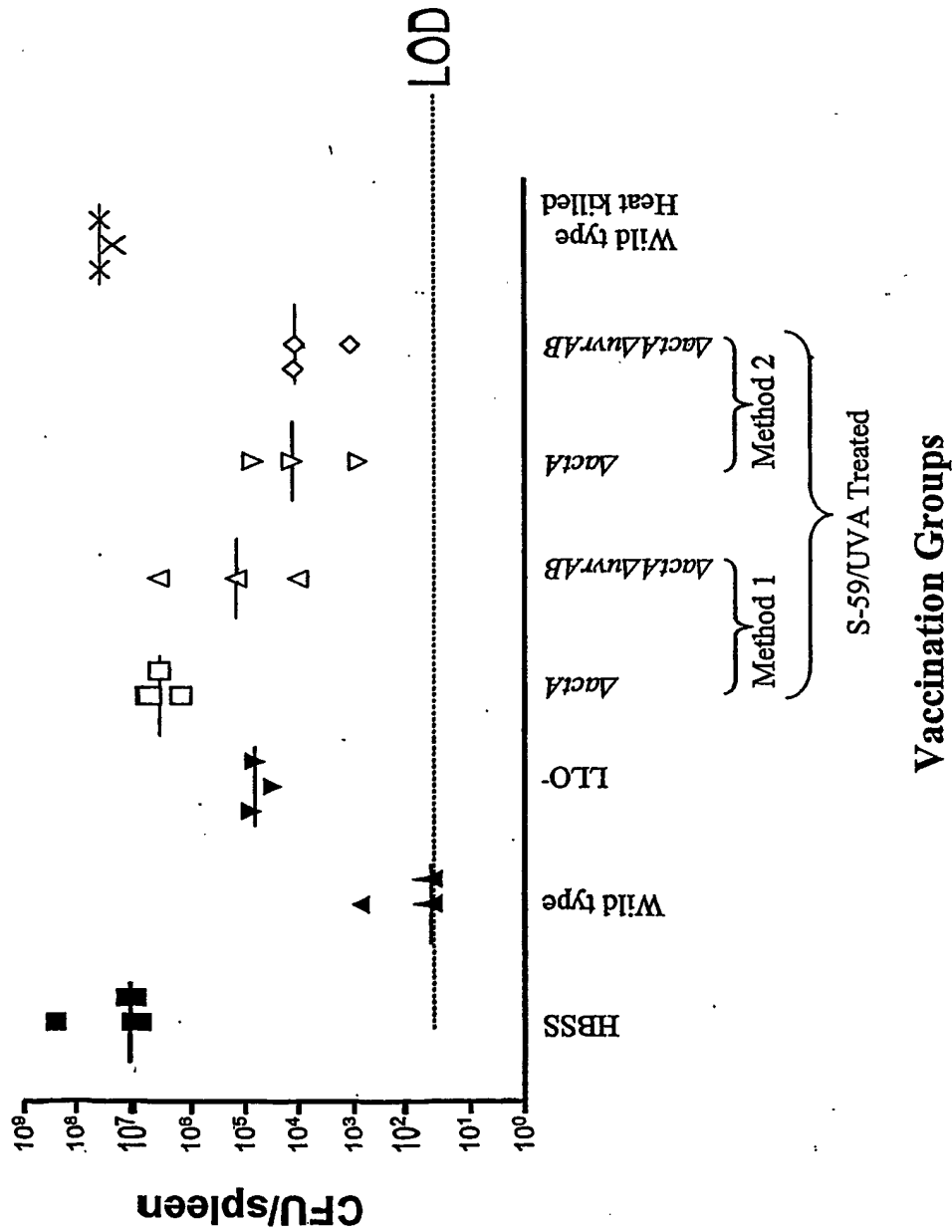


Figure 13A



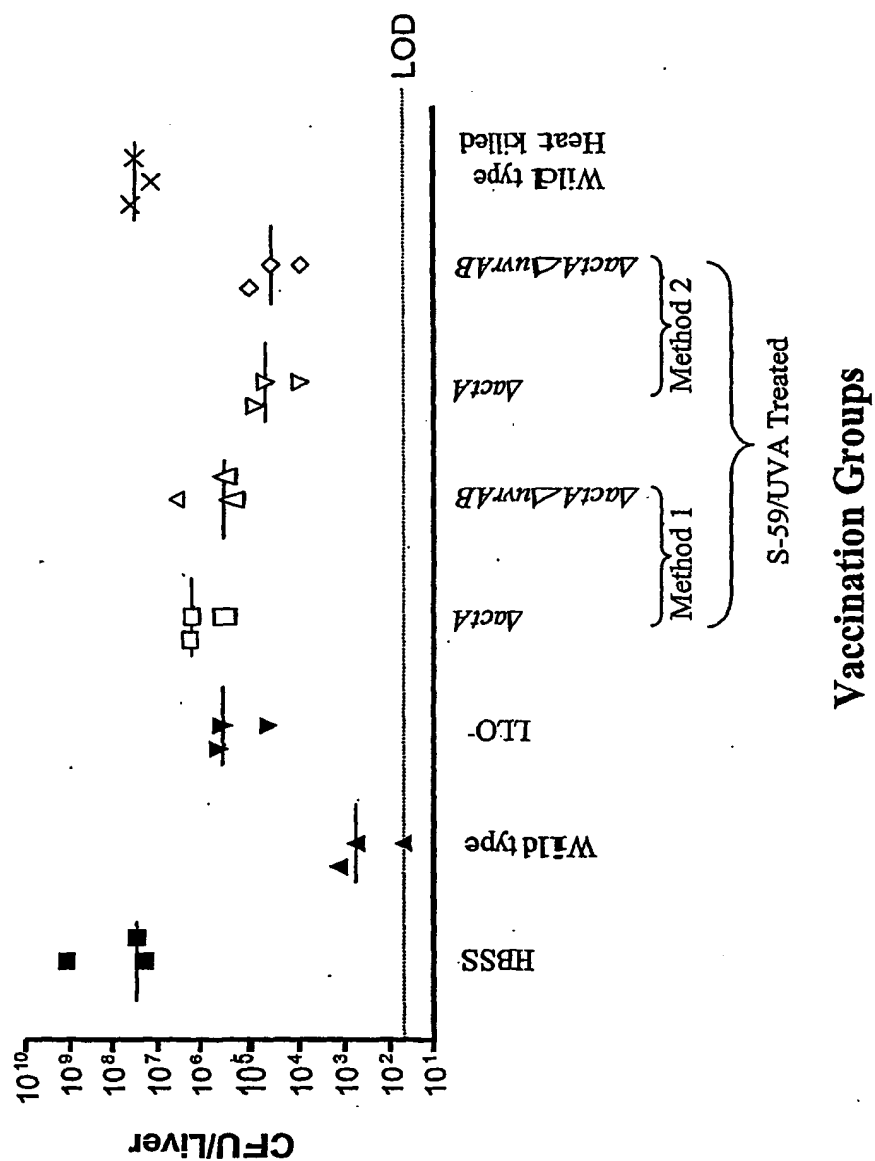


Figure 13B

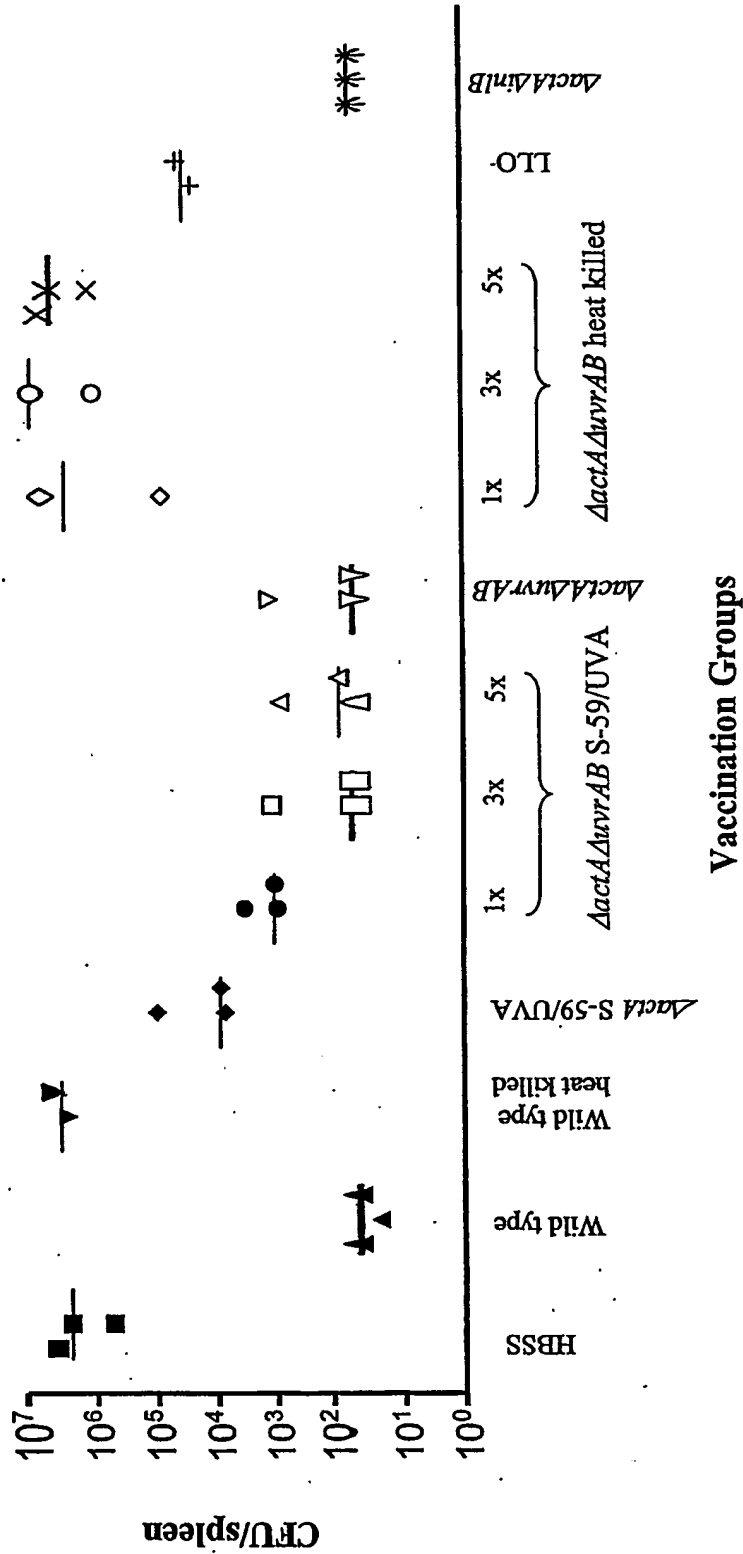


Figure 14A

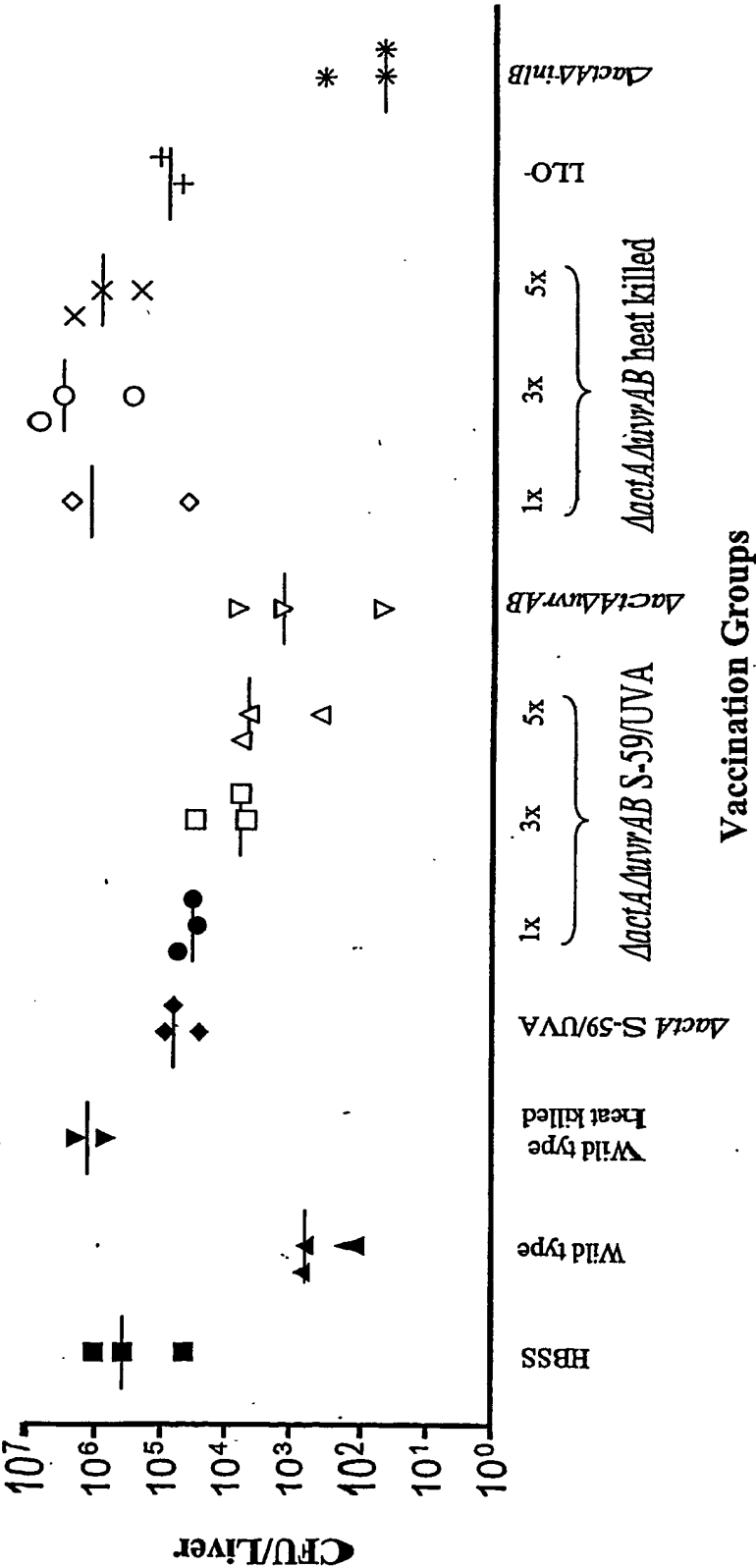


Figure 14B

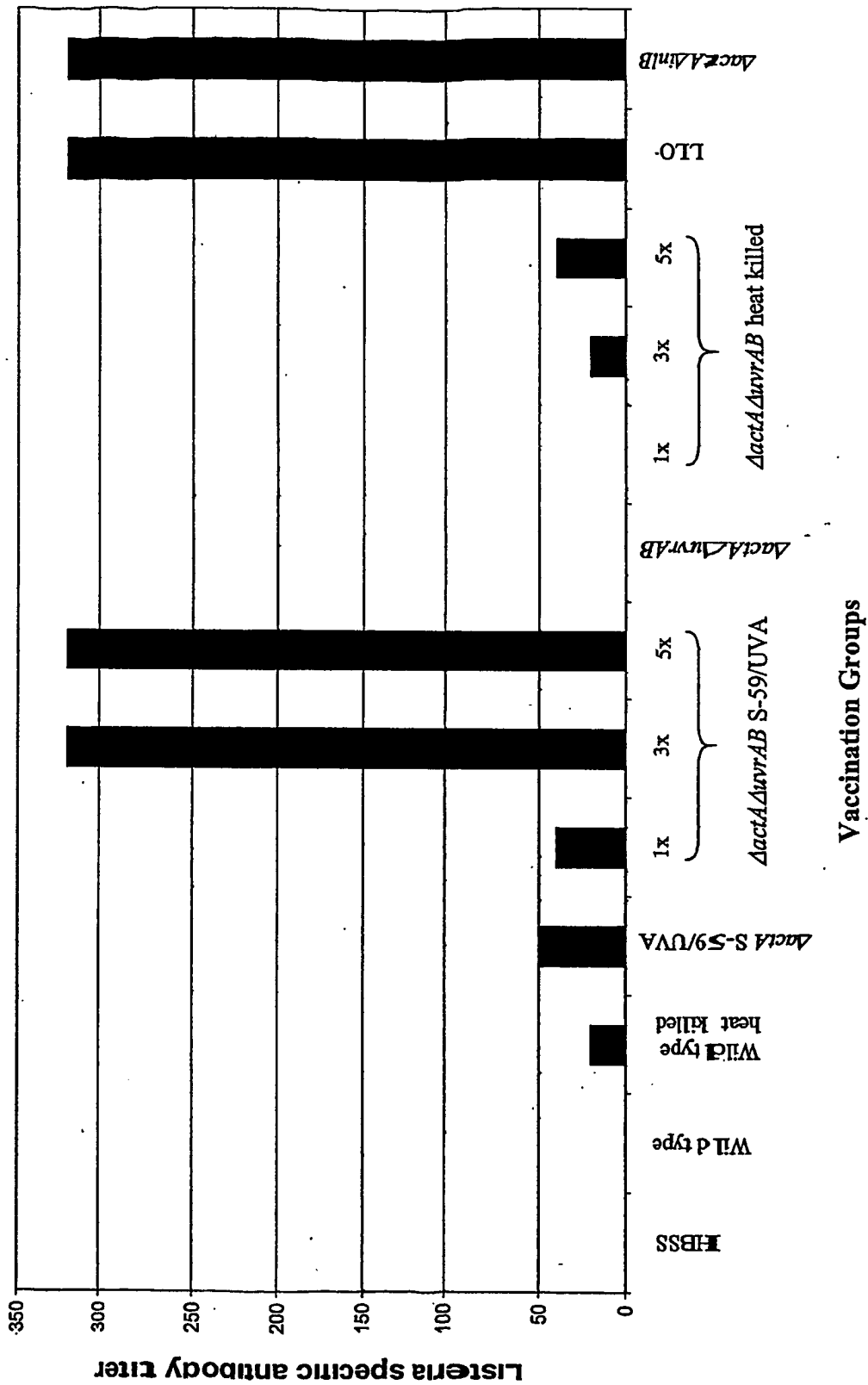


Figure 15

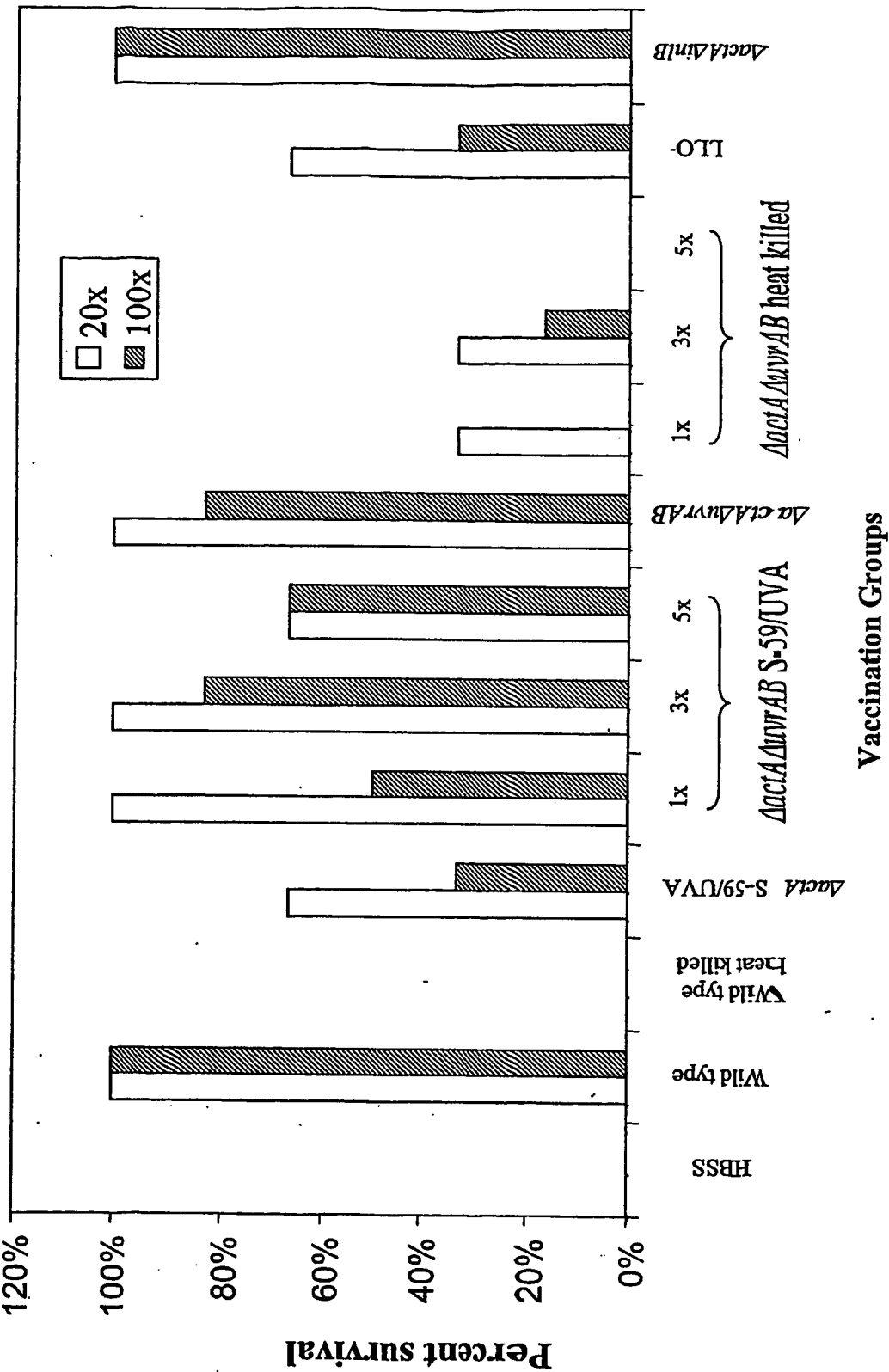


Figure 16

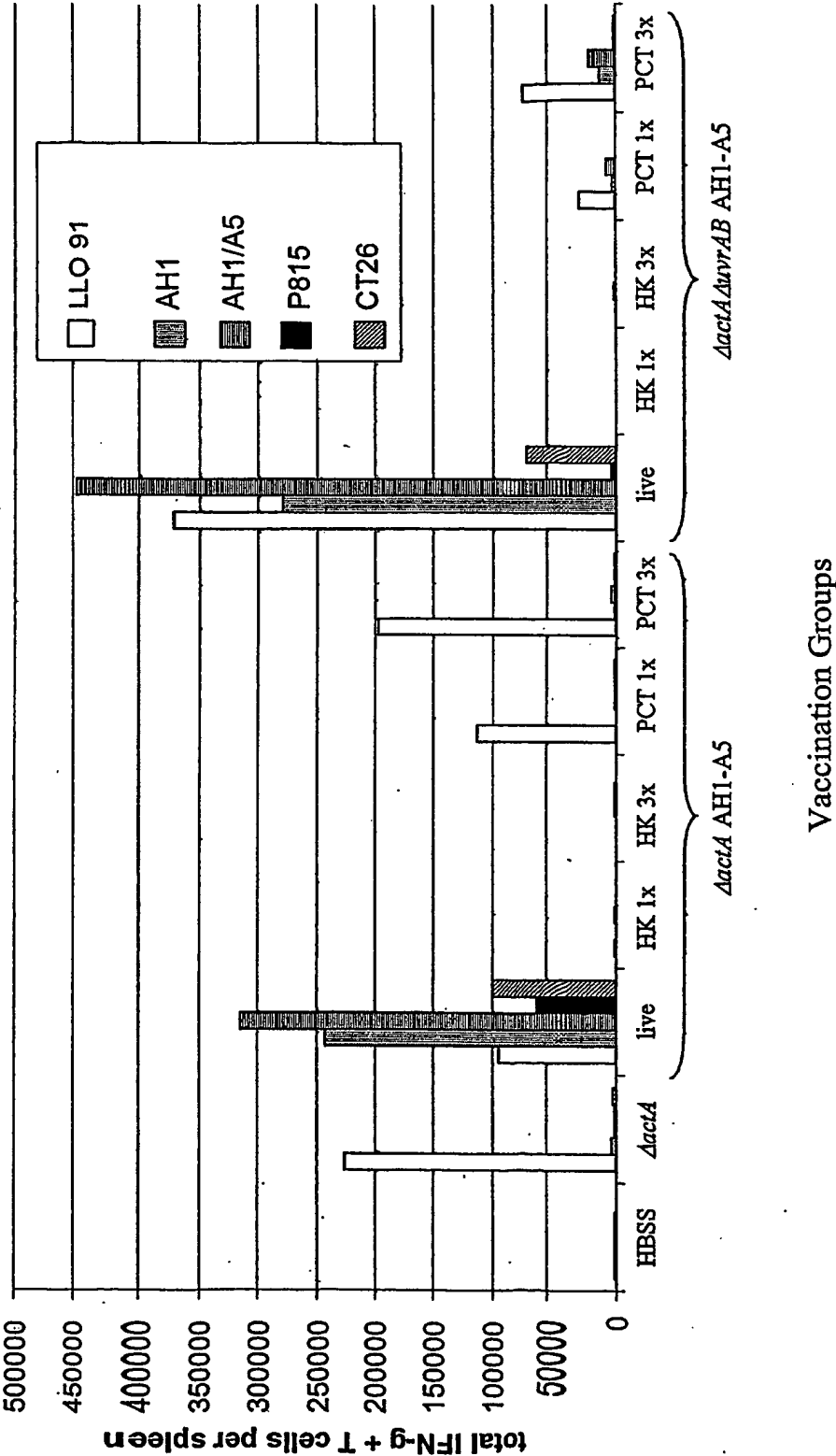


Figure 17

# ELISPOT for AH1/A5-specific T cells

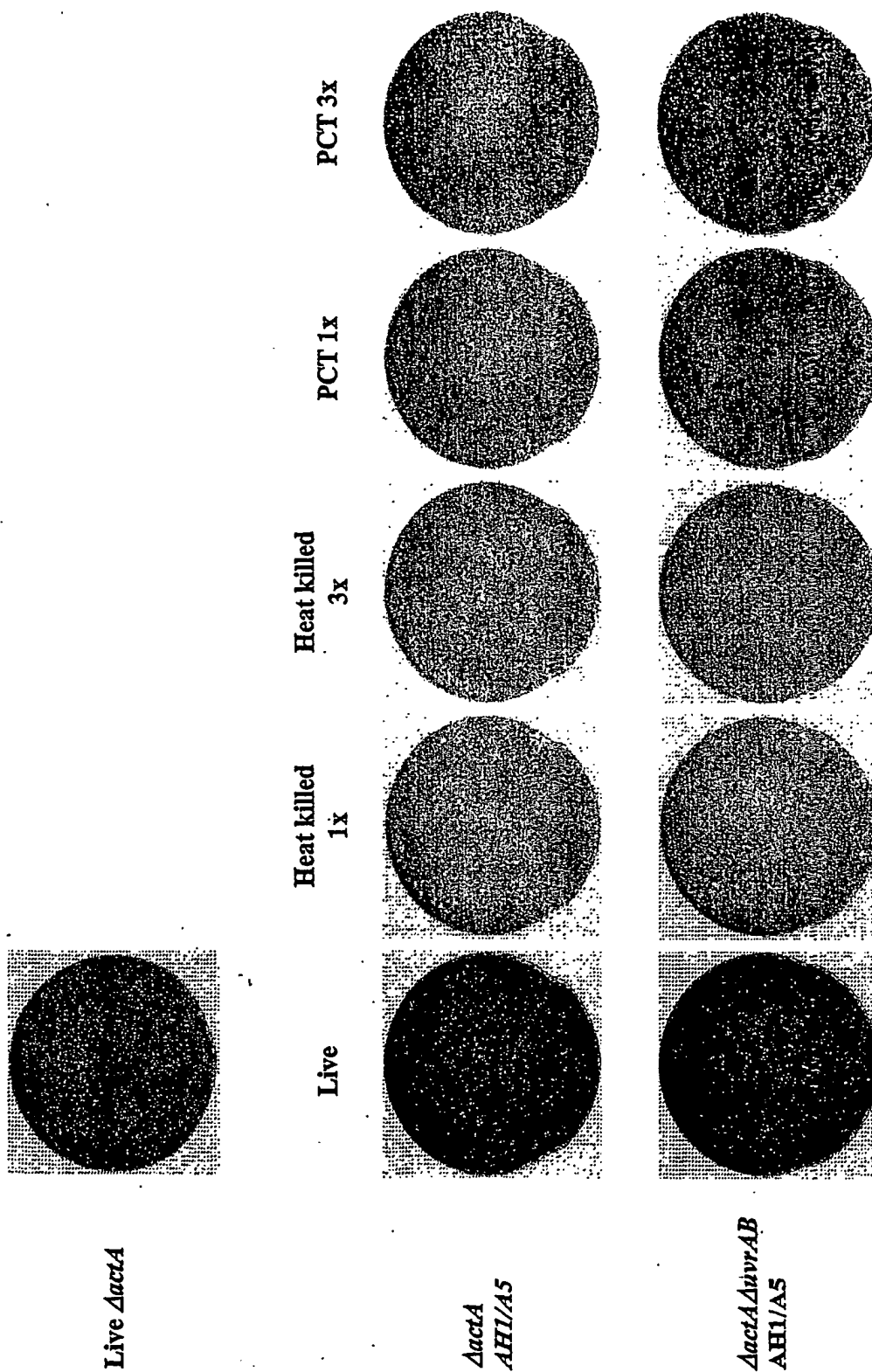


Figure 18A

# ELISPOT for AH1-specific T cells

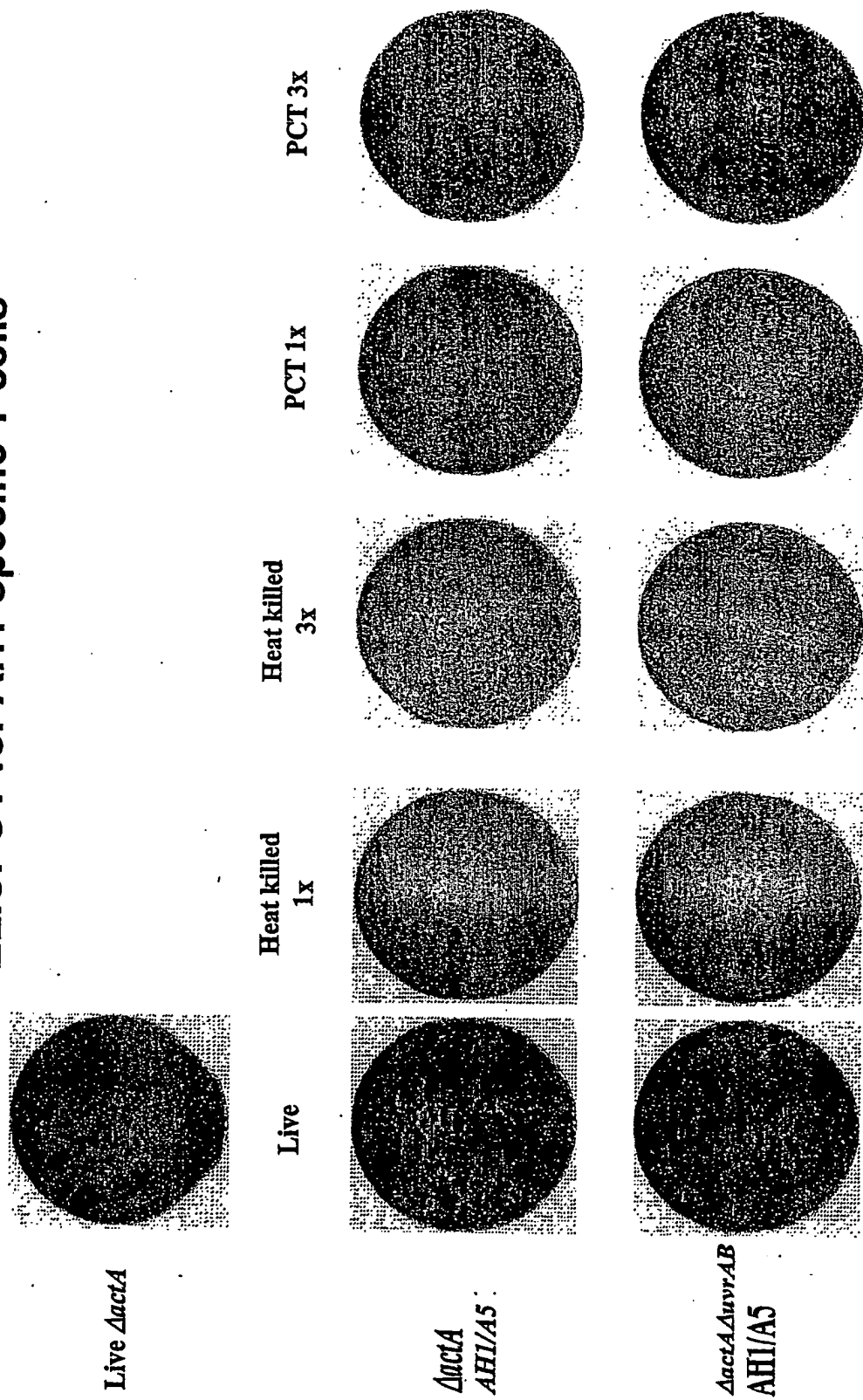


Figure 18B



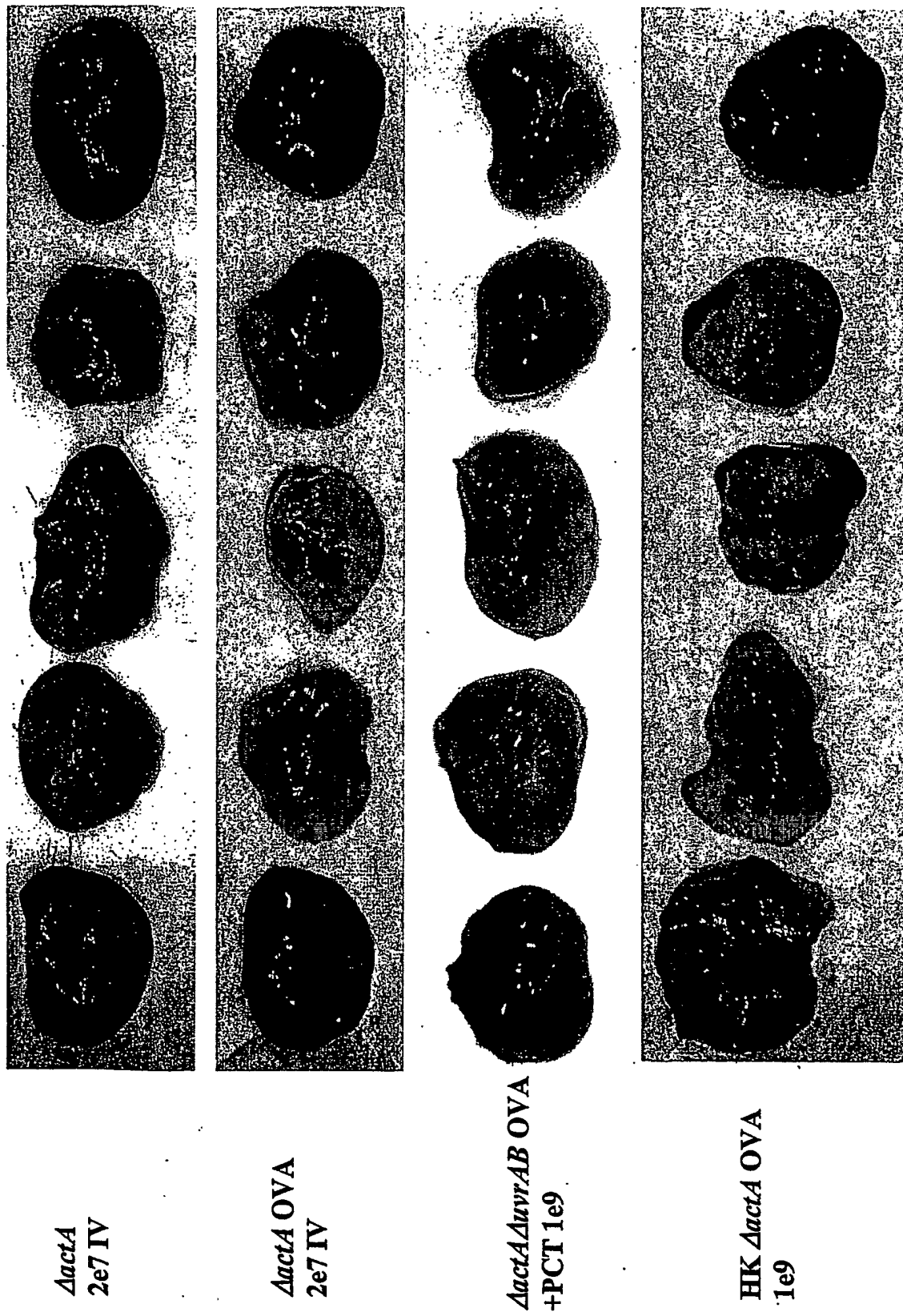


Figure 19A

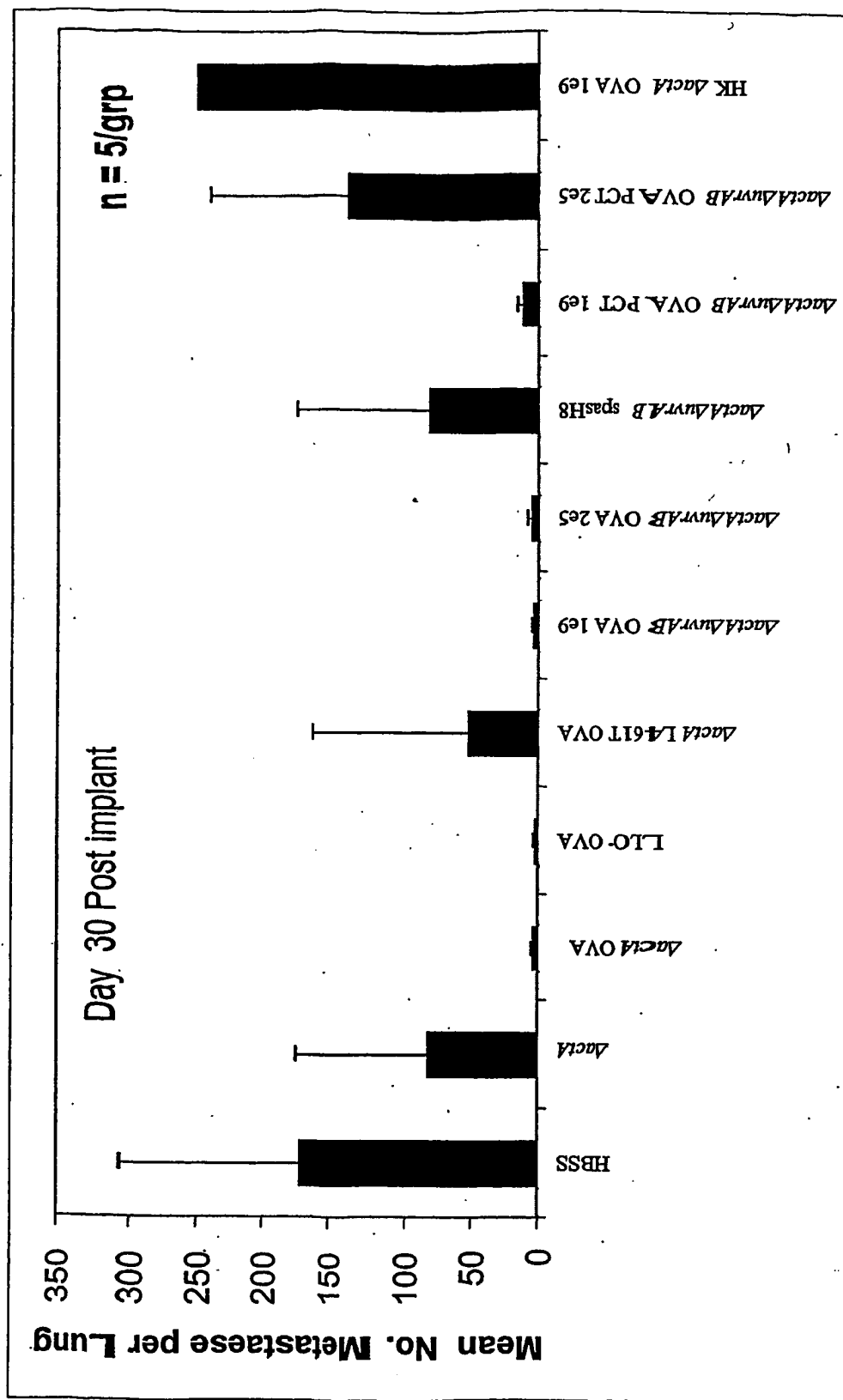


Figure 19B

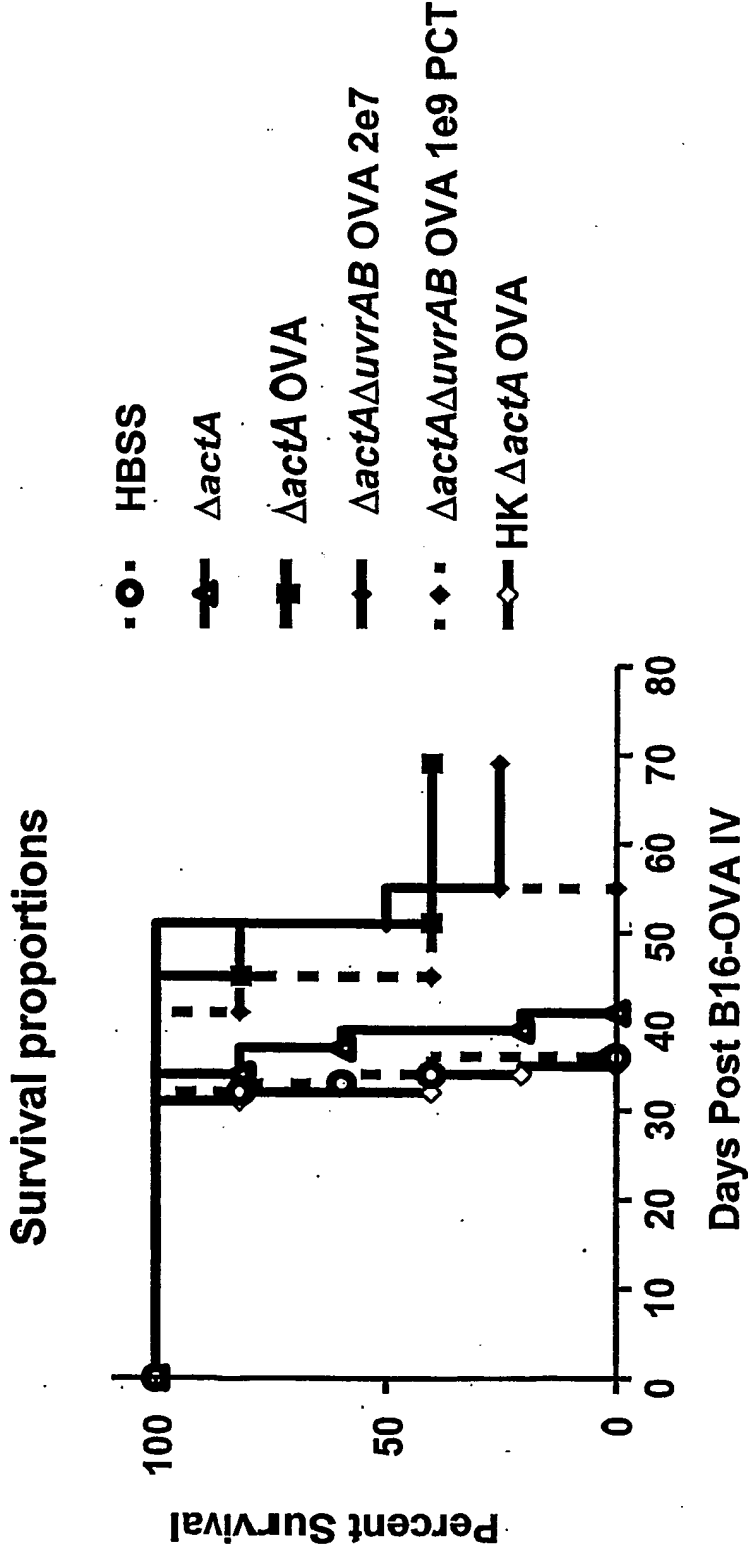


Figure 19C

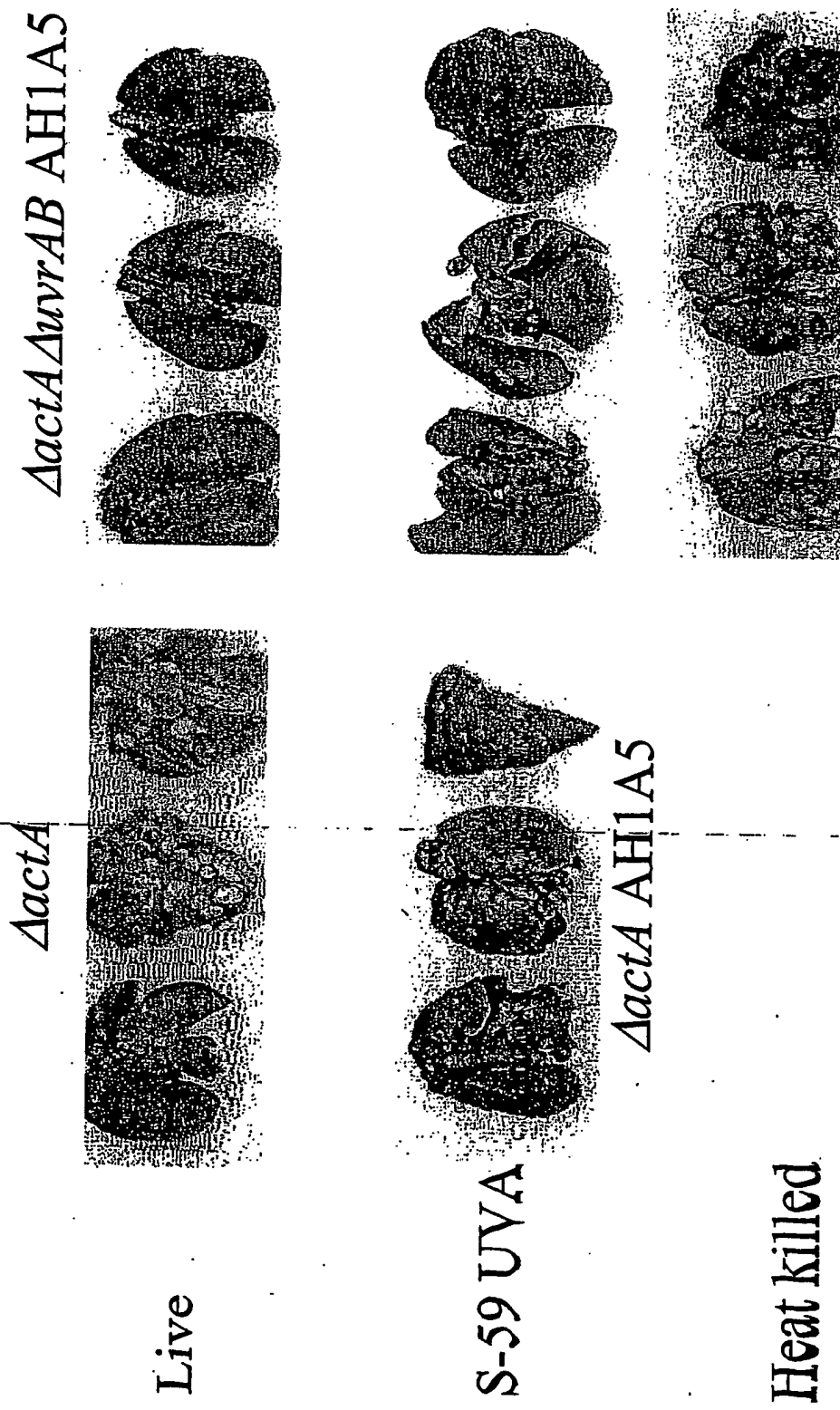


Figure 20A

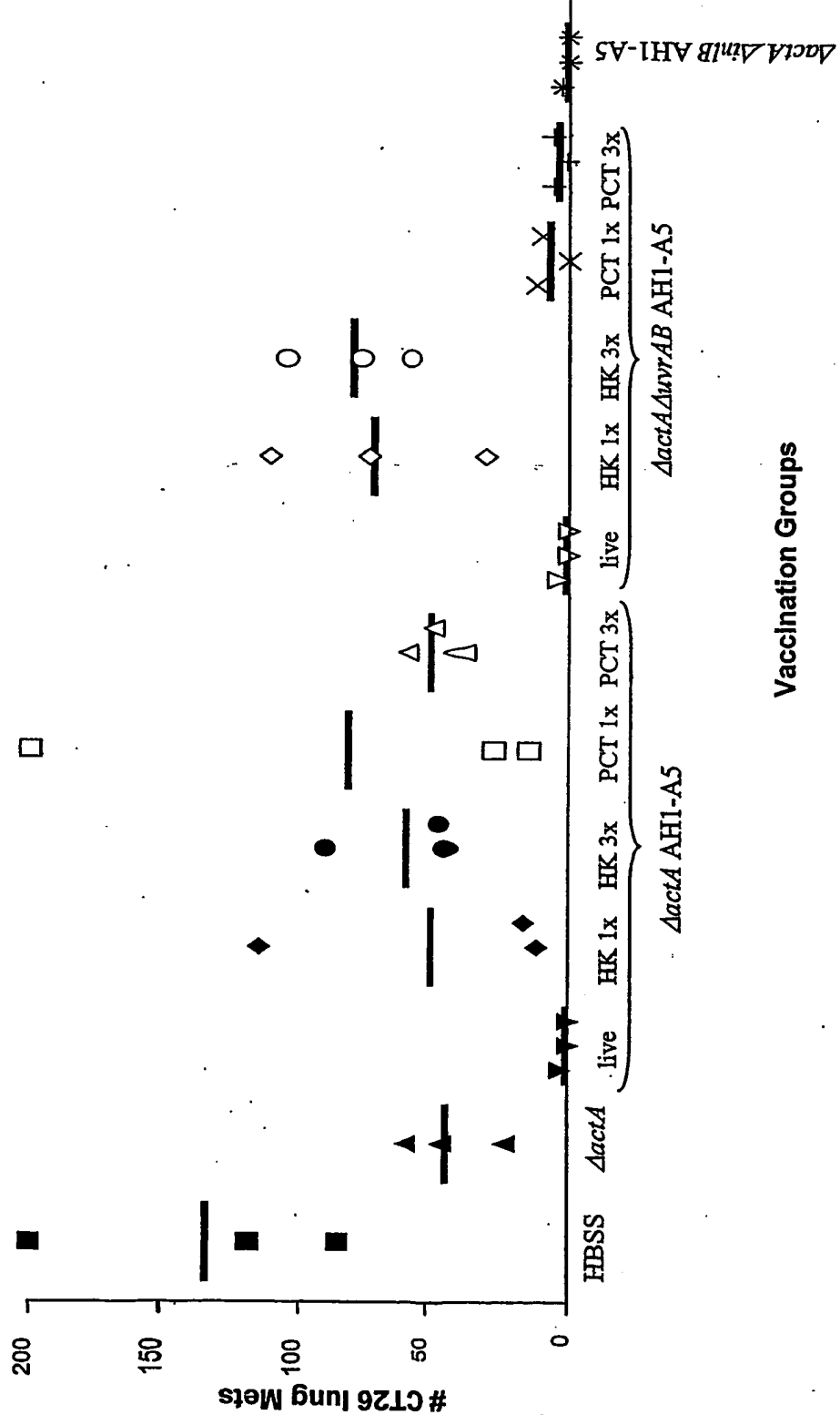


Figure 20B

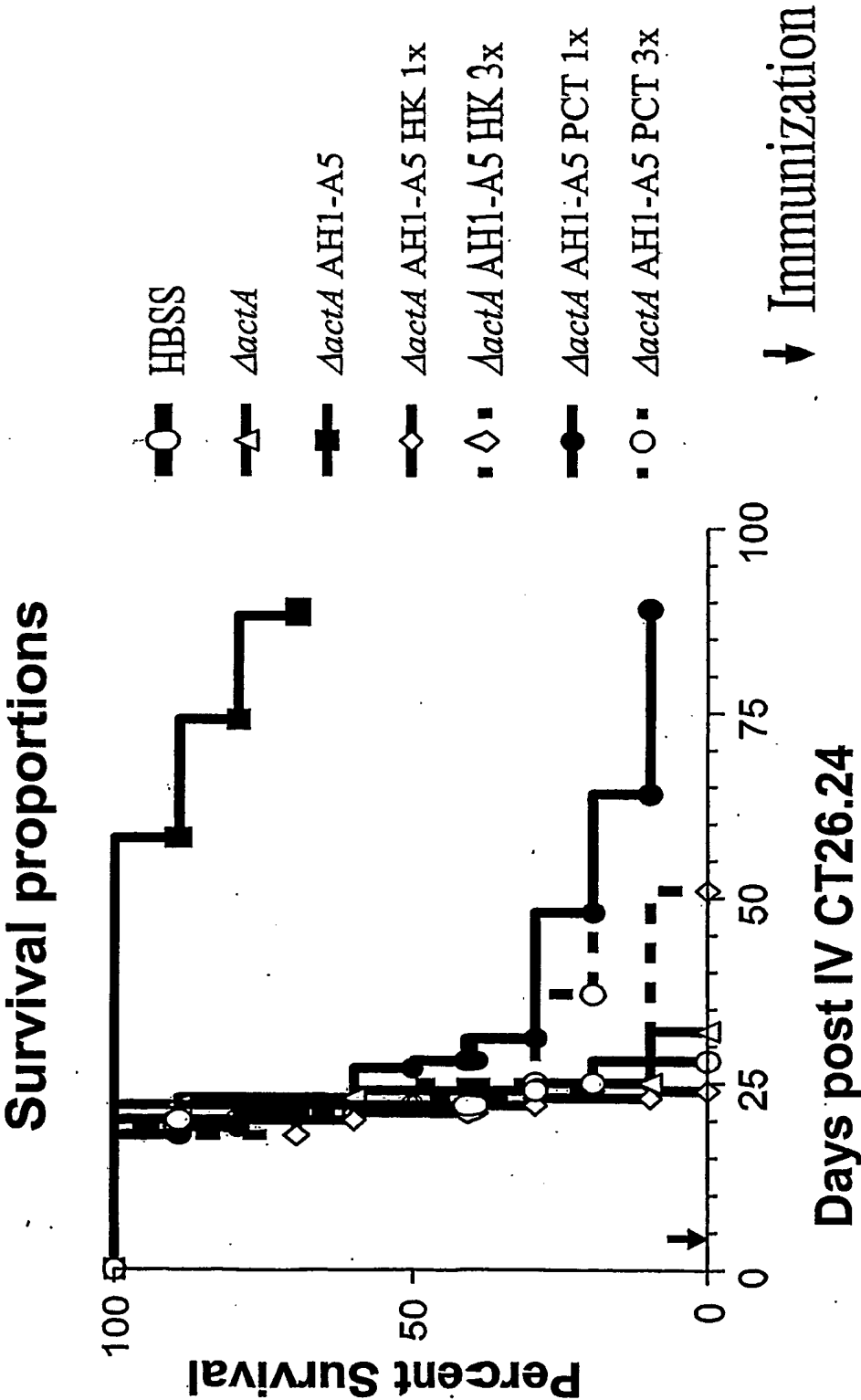


Figure 20C

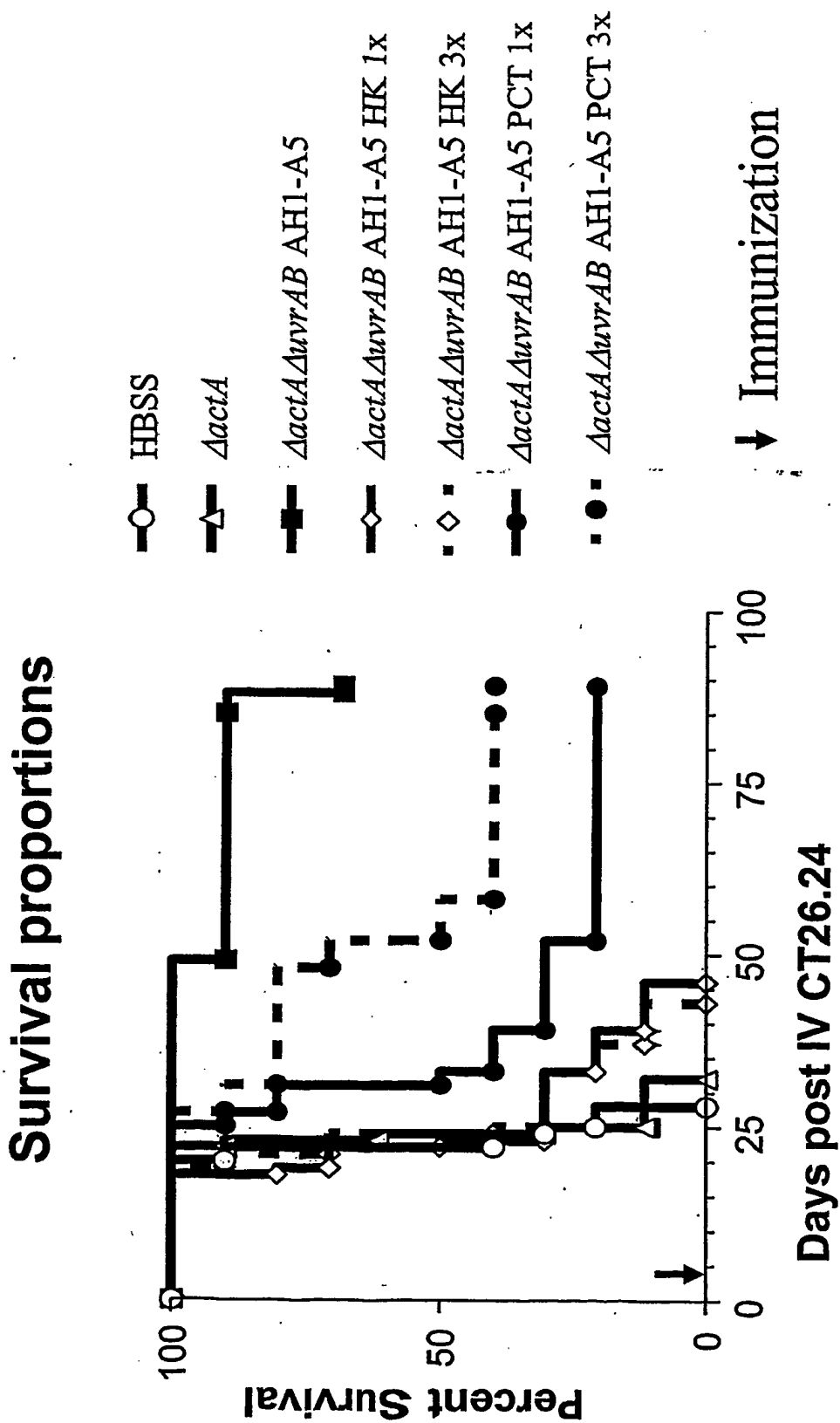
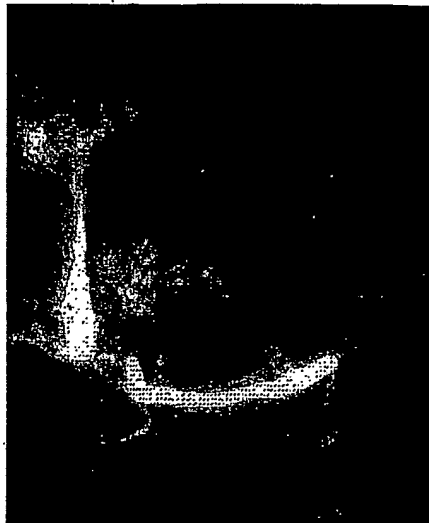
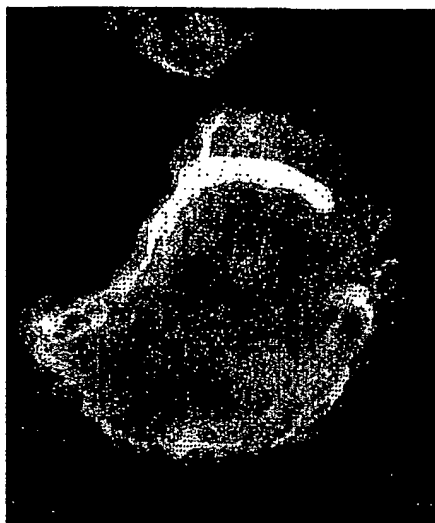


Figure 20D

RHODAMINE IMAGE



MERGED IMAGE



Listeria in phagolysosome,  
not detected in Rhodamine image

Figure 21A



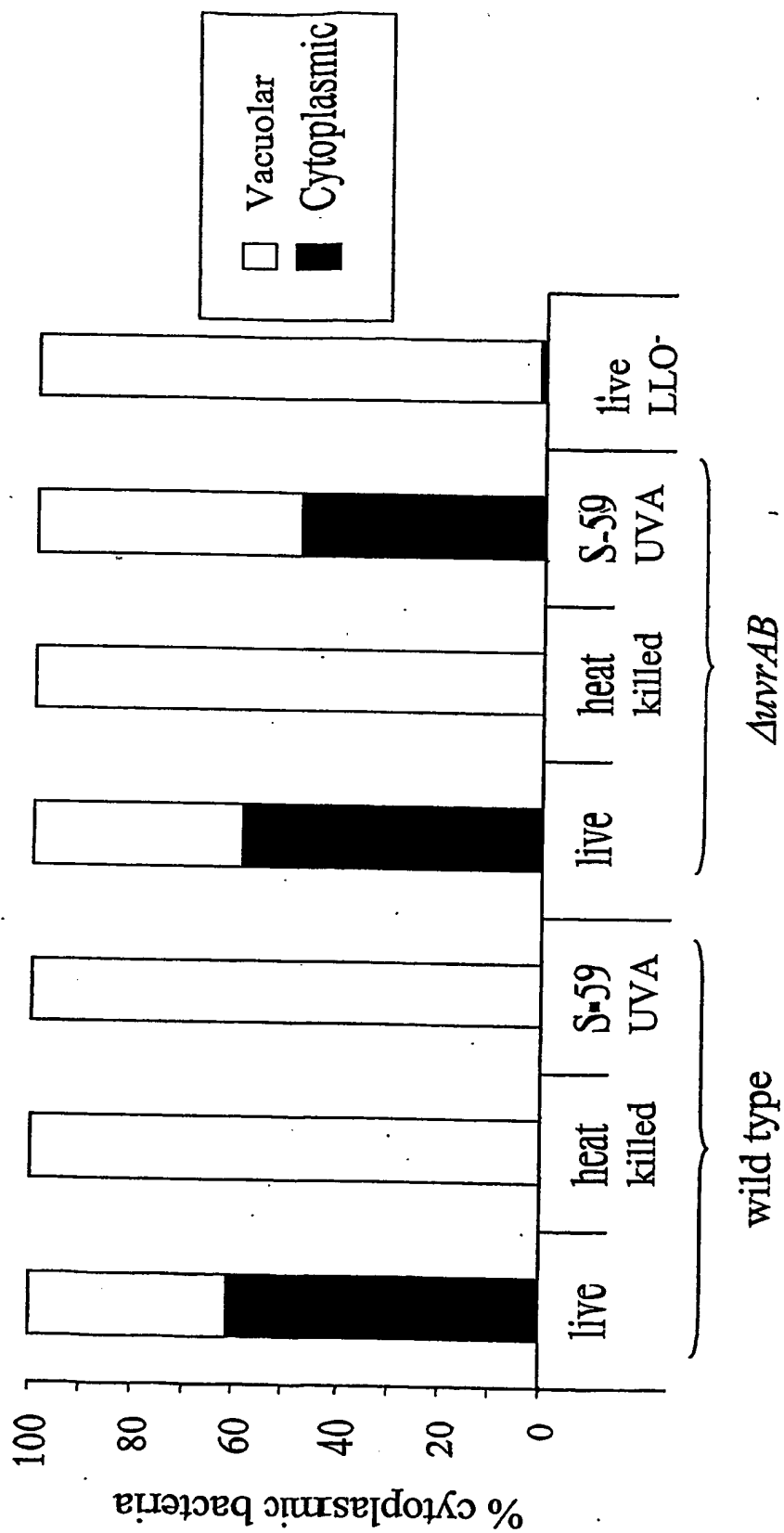


Figure 21B

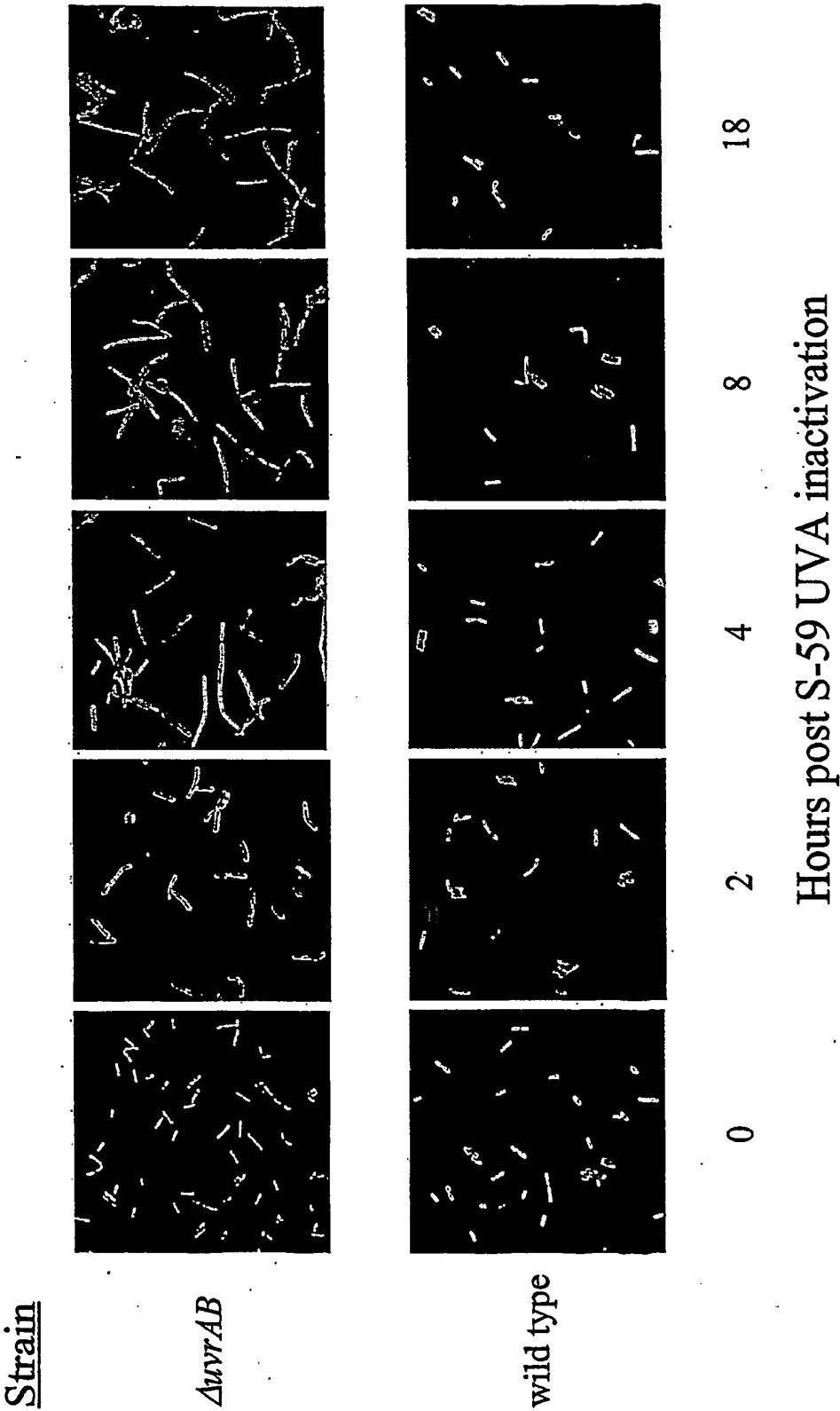


Figure 22

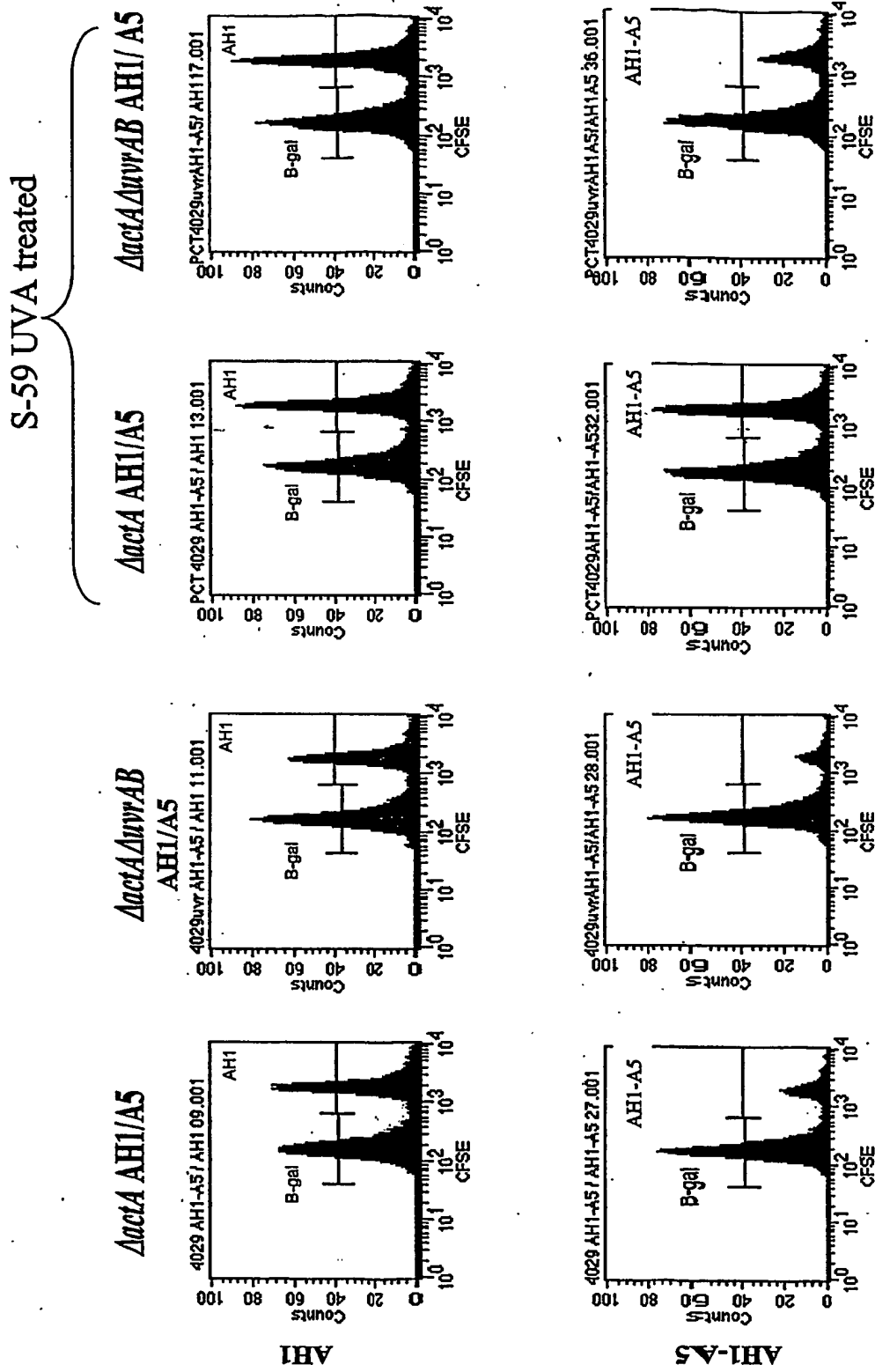
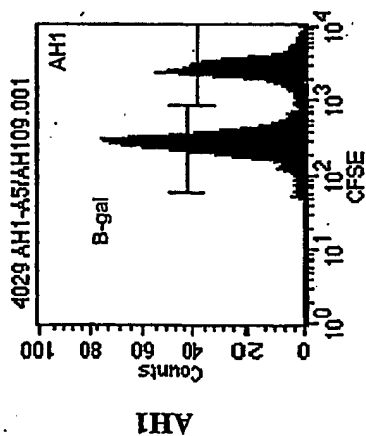


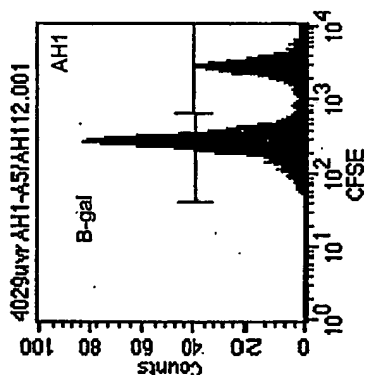
Figure 23A

S-59 UVA treated

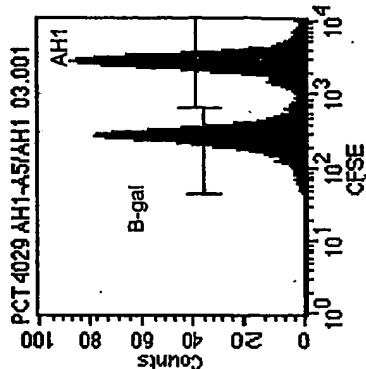
*ΔactA* AH1/A5



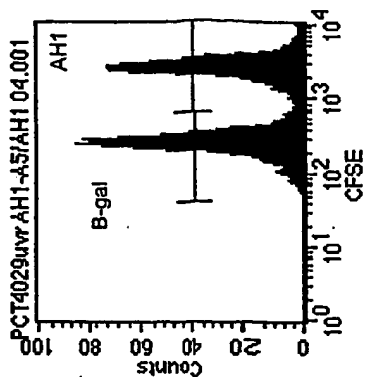
*ΔactA ΔuvrAB* AH1/A5



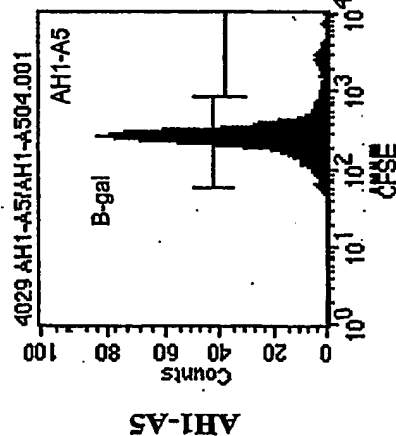
*ΔactA* AH1/A5



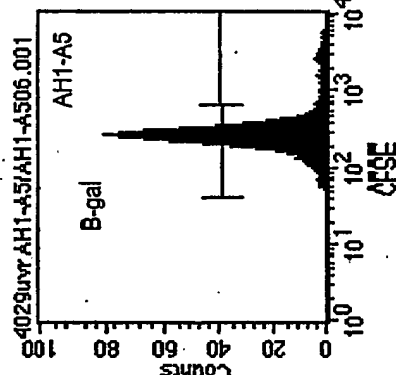
*ΔactA ΔuvrAB* AH1/A5



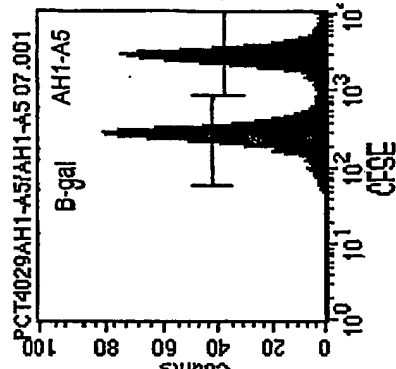
4029 AH1-Δ5/AH1-Δ504.001



4029uvr AH1-Δ5/AH1-Δ506.001



PCT4029AH1-Δ5/AH1-Δ507.001



PCT4029uvr AH1-Δ5/AH1-Δ510.001

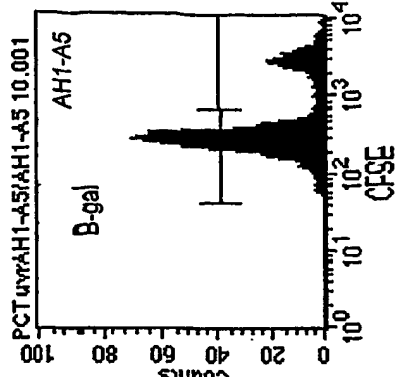


Figure 23B

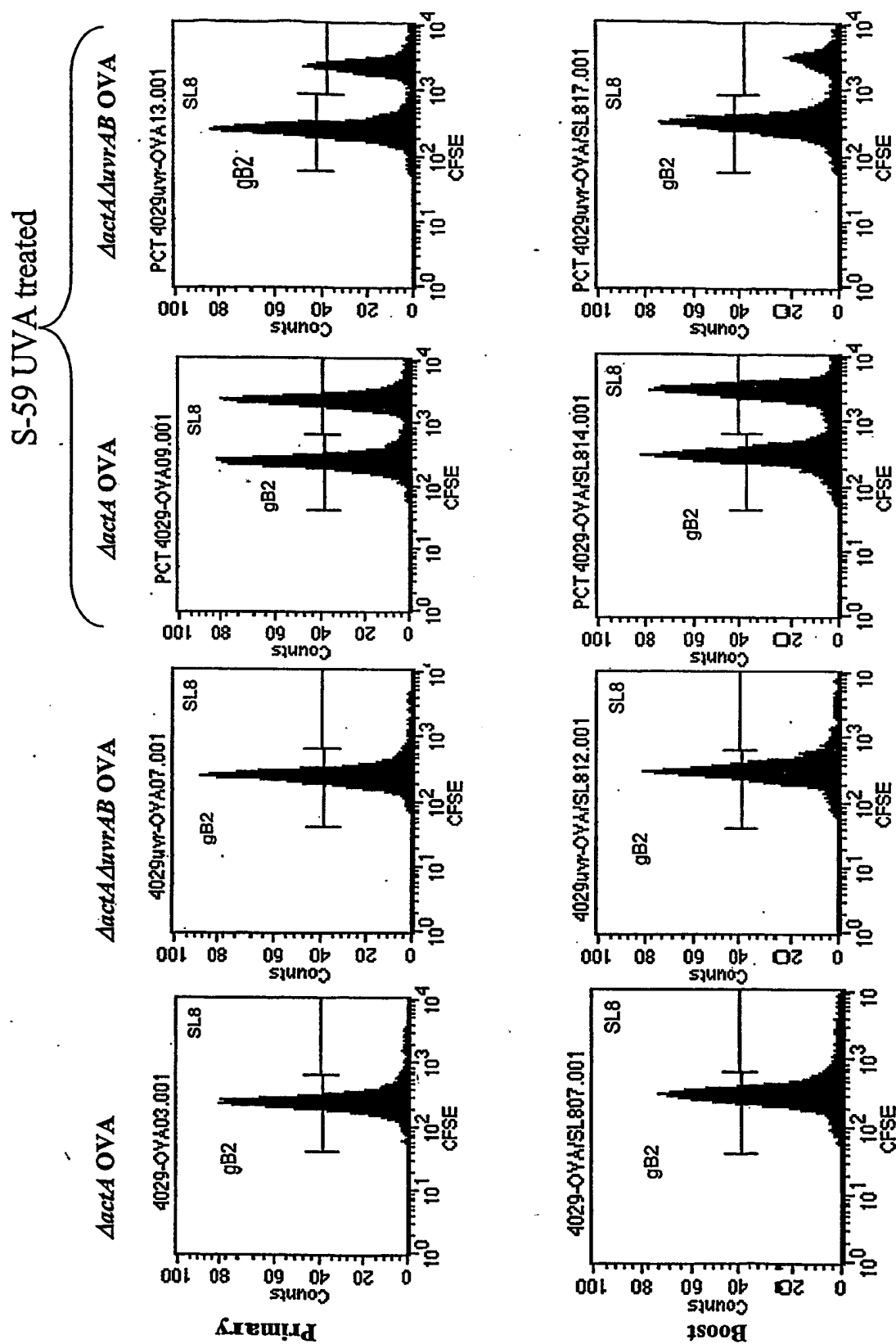


Figure 23C

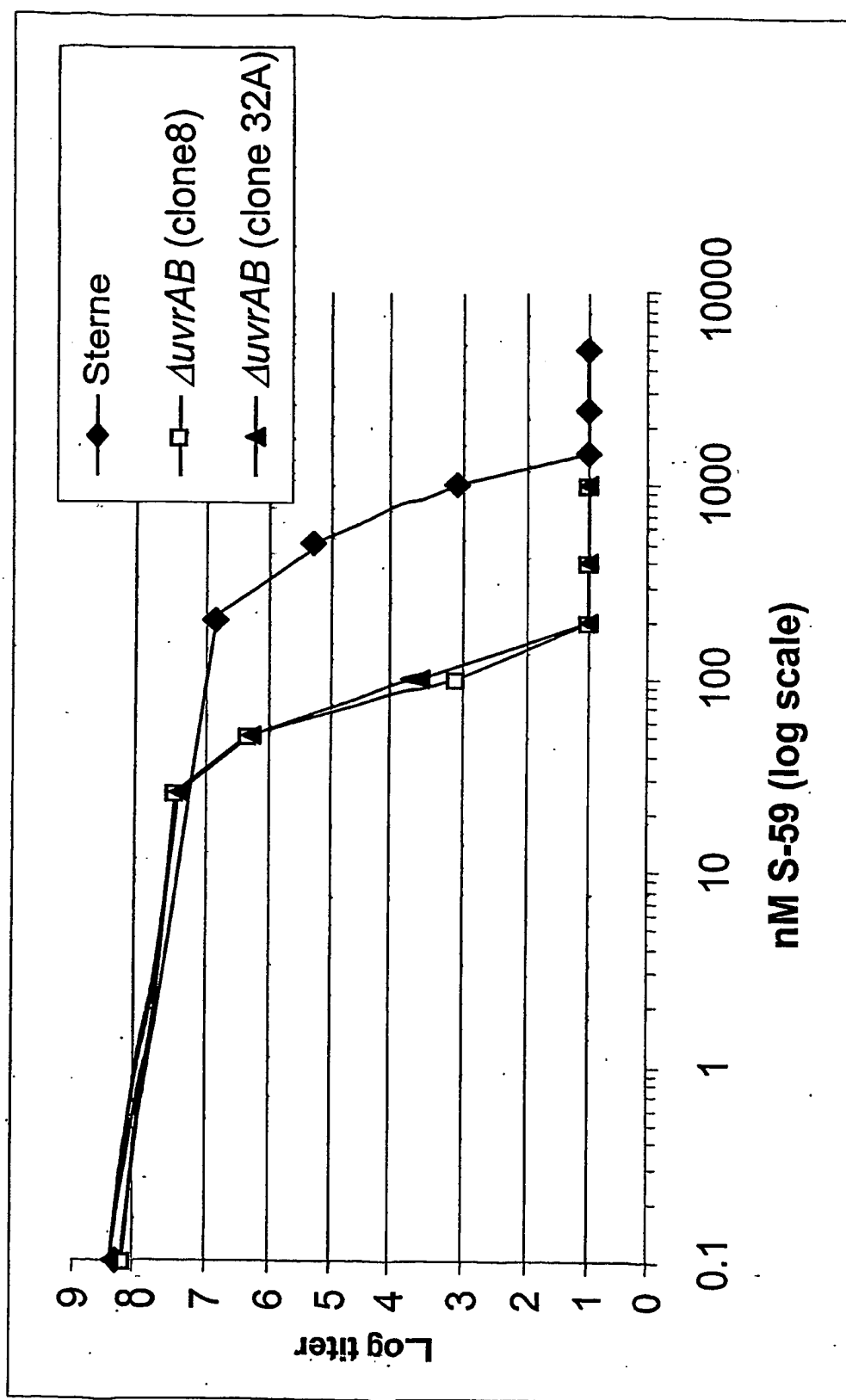


Figure 24

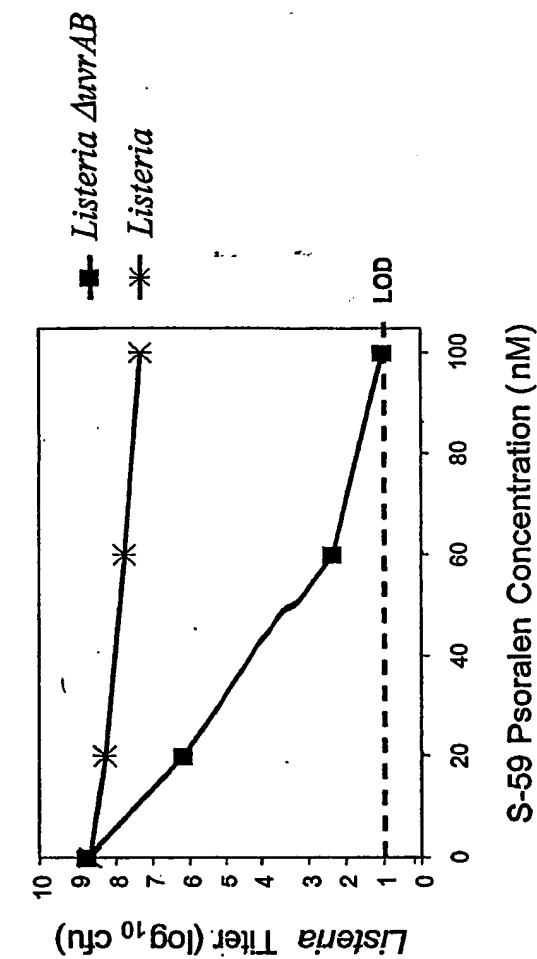
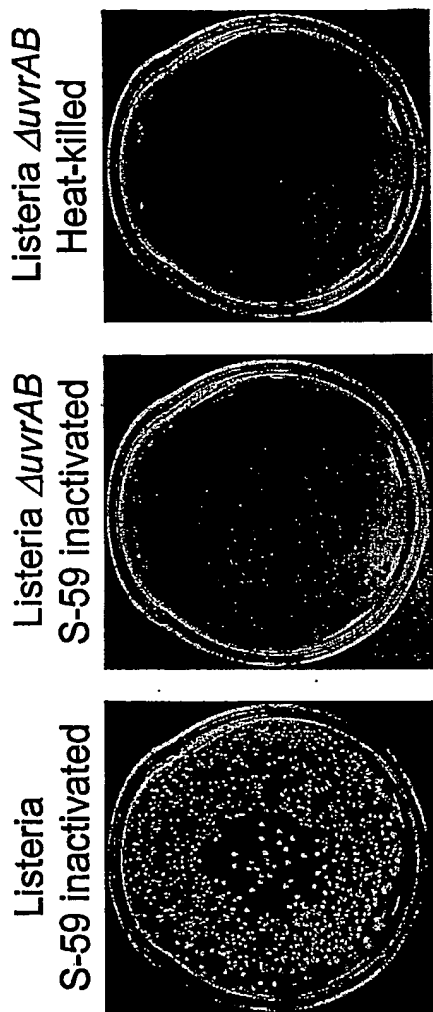


Figure 25

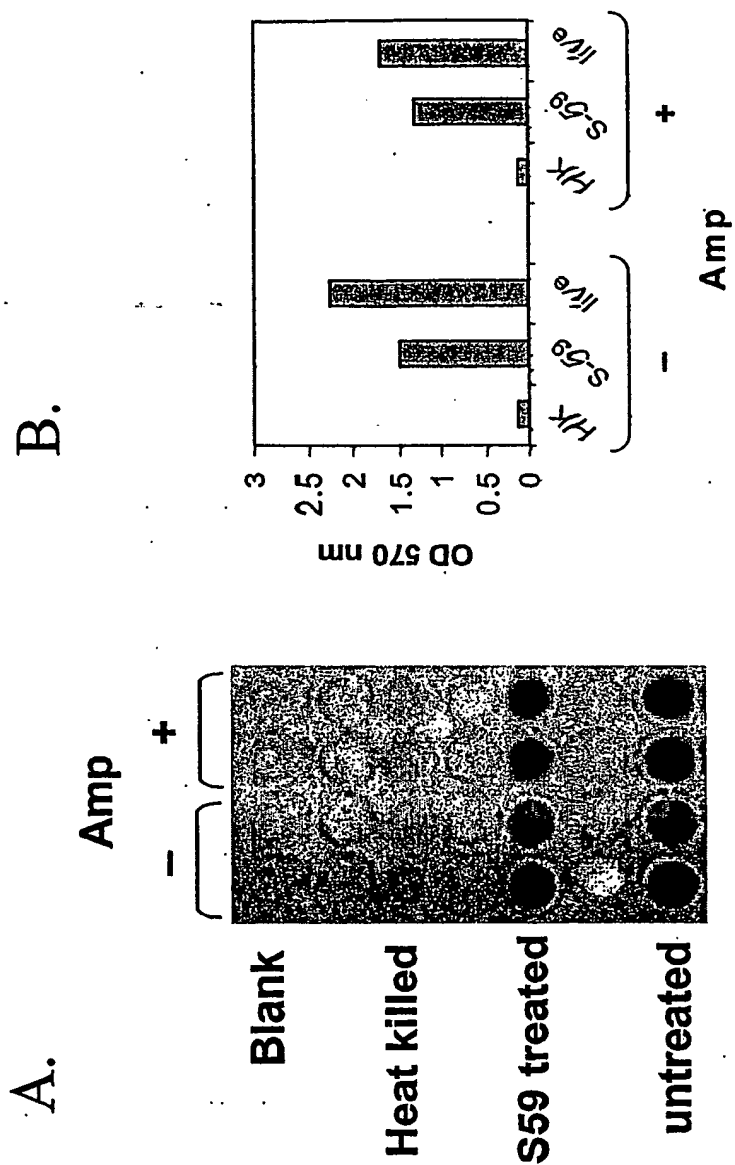


Figure 26

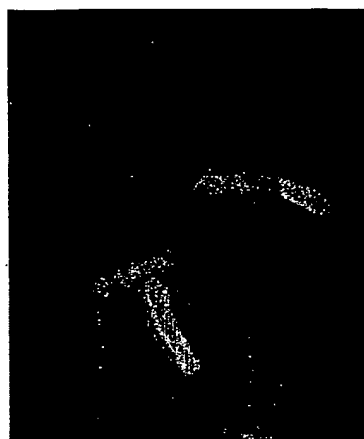




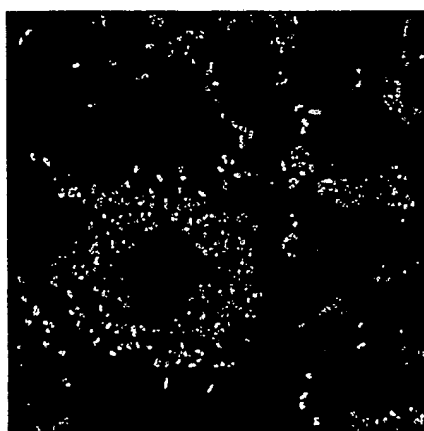
C. *Listeria*  $\Delta hly$  (LLO<sup>-</sup>)



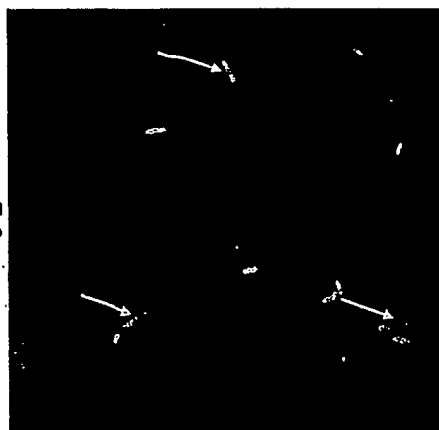
B. Wild type



E. *Listeria*  $\Delta uvrAB$   
S-59 UVA inactivated



A. Wild type



D. *Listeria*  $\Delta uvrAB$   
S-59 UVA inactivated

Figure 27

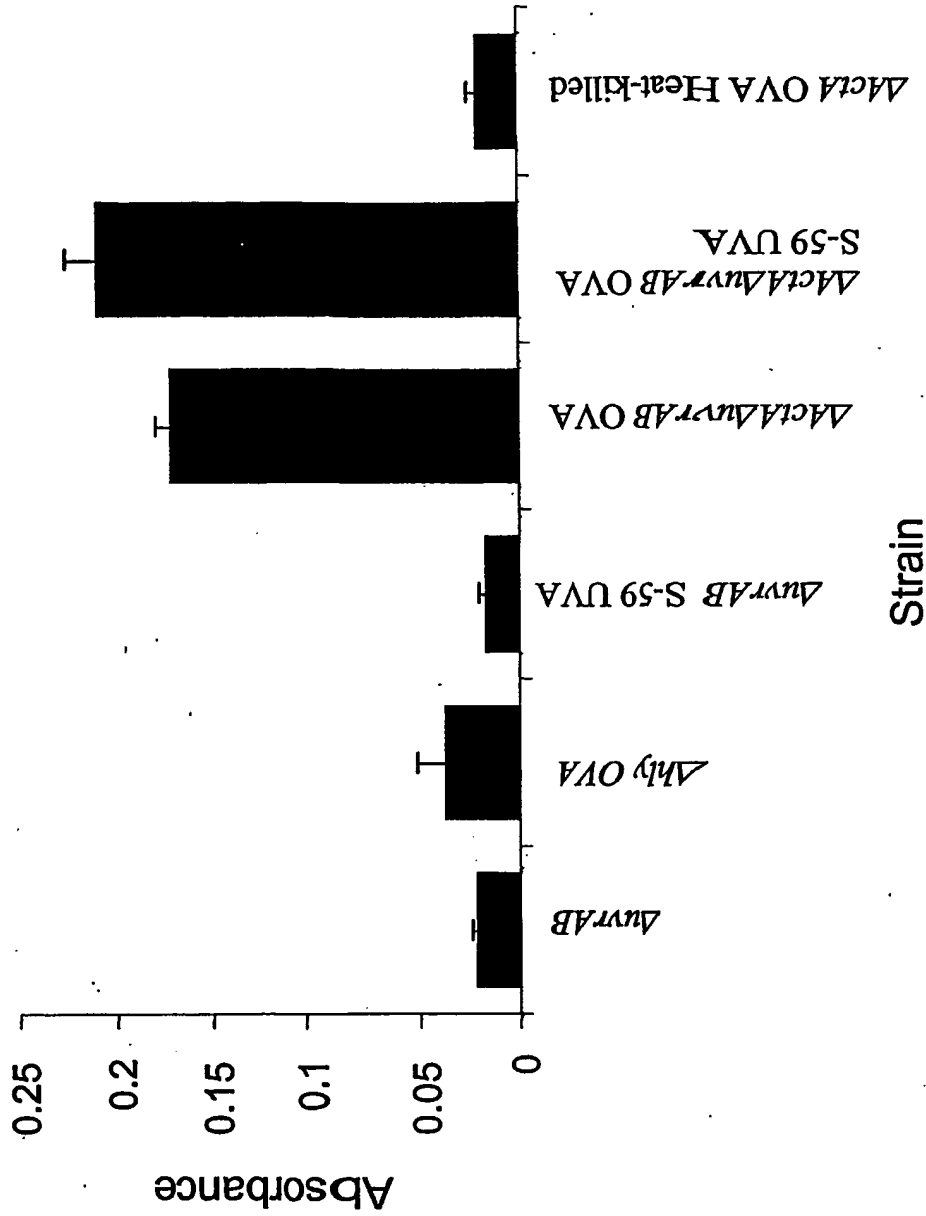


Figure 28

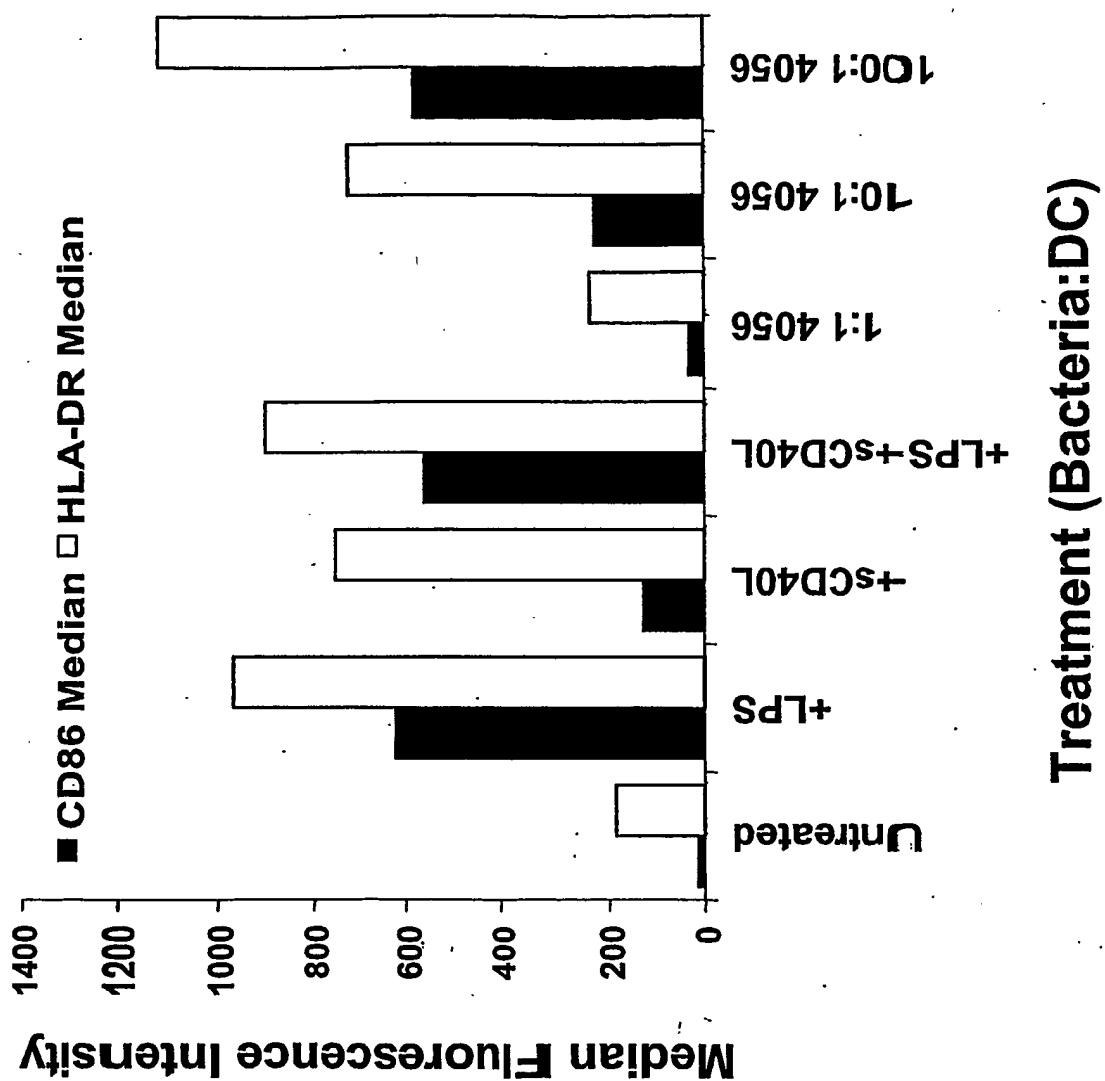


Figure 29A

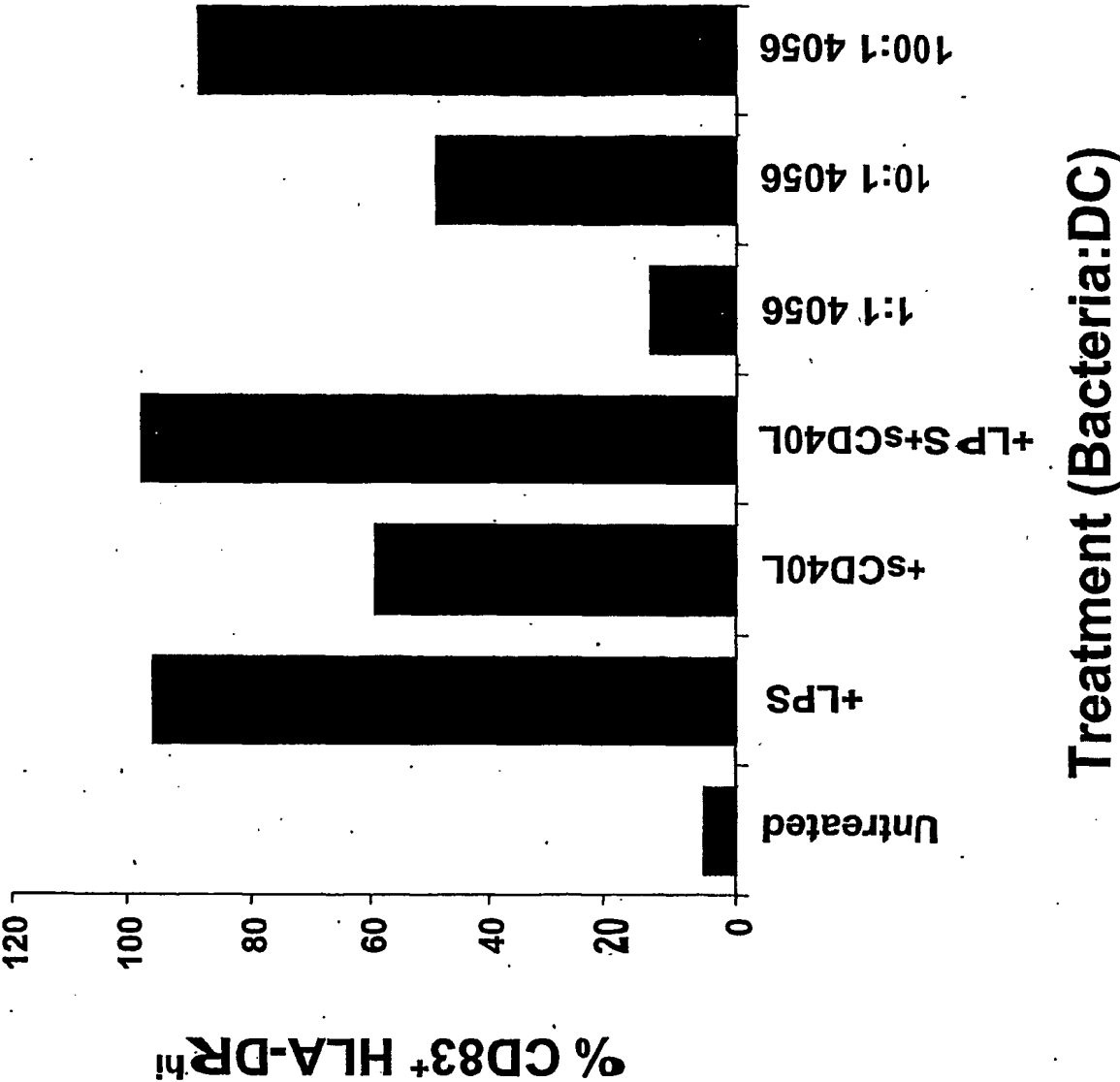


Figure 29B

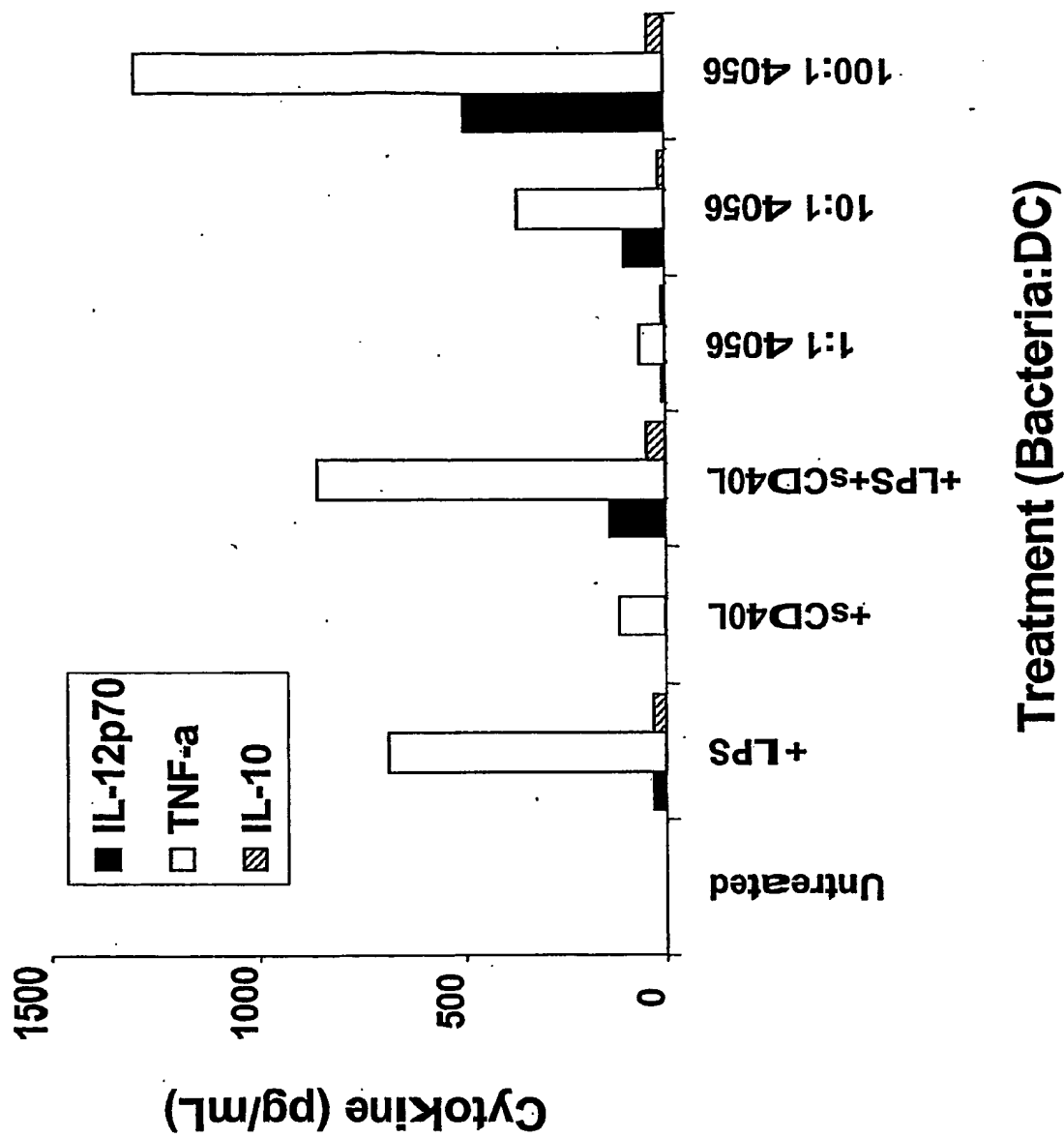
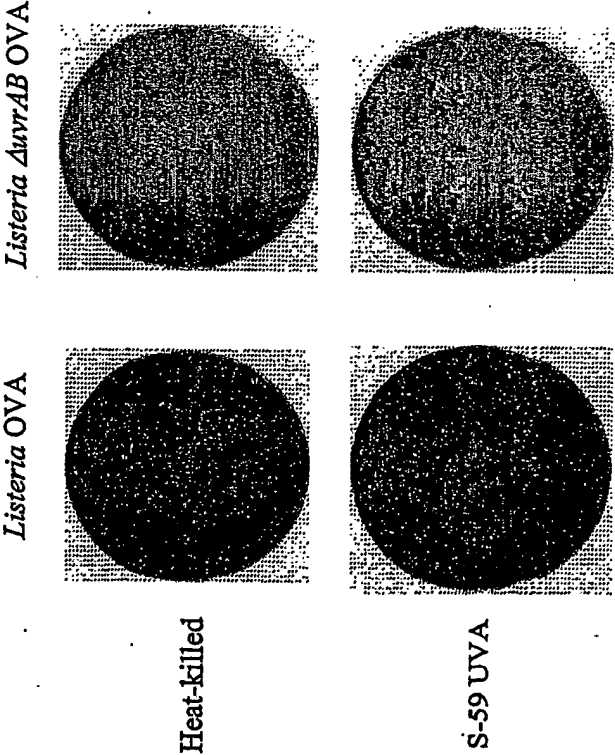
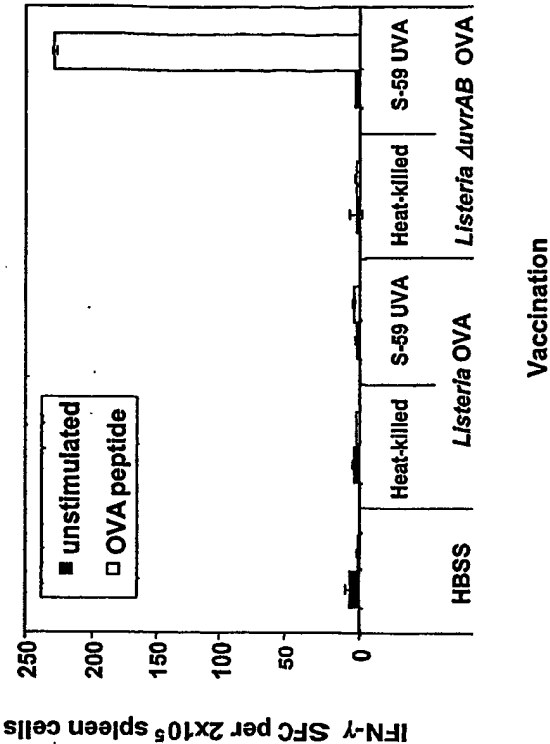


Figure 29C



A.



B.

Figure 30

**LLO<sub>(ss-PEST)</sub>-OVA/PR3 class I  
Primary Amino Acid Sequence**

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 V E S Q T N G I I R N V L Q P S S V D S Q T A M V L V N A I V F K G L W E K T F K  
 D E D T Q A M P F R V T E Q E S K P V Q M Y Q I G L F R V A S M A S E K M K I  
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 I T D V F S S S A N L S G I S S A E S L K I S Q A V H A A H A E I N E A G R E V V G  
 S A E A G V D A A S V S E E F R A D H P F L F C I K H I A T N A V L F F G R C V S  
 P

**S I I N F E K L:** OVA H-2 K<sup>b</sup> epitope

**V L Q E L N V T V:** PR3 HLA A-2 restricted class I epitope (a.k.a. PR1)

Listeria secA1 signal peptide and PEST sequence is underlined

Figure 31

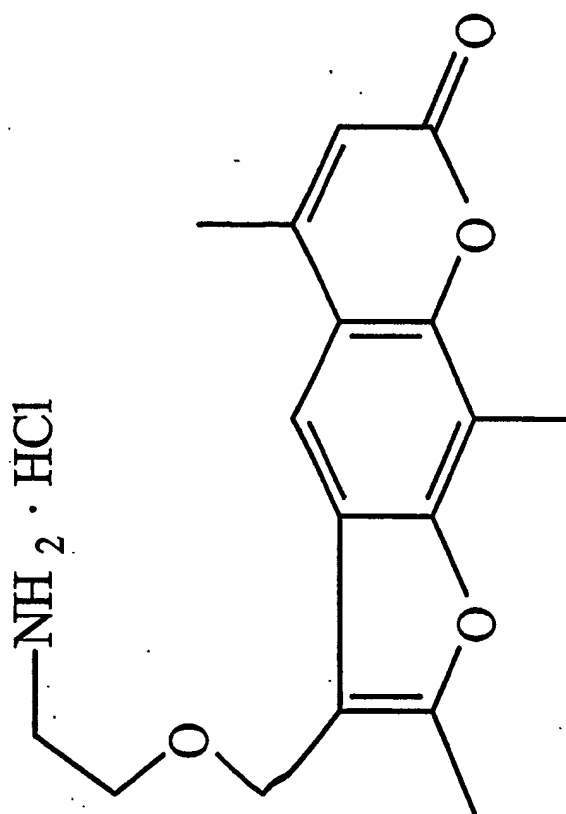


Figure 32



## SEQUENCE LISTING

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 DUBENSKY Jr., Thomas W.  
 BROCKSTEDT, Dirk G.  
 BAHJAT, Keith  
 HEARST, John E.  
 COOK, David

<120> MODIFIED FREE-LIVING MICROBES, VACCINE  
 COMPOSITIONS AND METHODS OF USE THEREOF

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 <141> 2004-02-06

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&lt;210&gt; 10

&lt;211&gt; 4612

&lt;212&gt; DNA

<213> *Listeria monocytogenes*

&lt;400&gt; 10

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<212> DNA

<213> *Listeria monocytogenes*

<400> 11

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&lt;211&gt; 1908

&lt;212&gt; DNA

<213> *Bacillus anthracis*

&lt;400&gt; 19

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cggctcctggc gttgctgaaa cgtaaataac ttgattcggt tt ctcttcaa actcatcaaa 780
tgt gagcgggt ctattatcta aagctgatgg cagacggaat cc atgatcca caagcacttg 840
ttacgcgct tggccccgct tatacatcgc tcttacttgc gg cactgata cgtgggactc 900
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accgttatca ttttaattcct ttaa acgctc ttctaattct tt ttcgatat tttcaatagc 1140
gac cttcatc ttttcttcac gtgt aaacgaa gtgagatgct gg gaagattg ctacatgatc 1200
acgttcttgc aatacttctc ccgt taaagc atttacttgc cg aatacgat caatttcac 1260
gccaaaaaac tcaattcgaa tgcaatgctc gtcaagtgat gc cgggaaga tttcaactac 1320
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aat aattaca tcatccggtt caaataatgc ggacgttgct ga gtagcgca atttatcgat 1560
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tggtgataa taatcgtaat aa ctaacaaa atattcaact gcattattcg ggaaaaagtc 1 680
tttcaactca ctatataact gt cctgctaa cgttttattg tgagccatga caagcggttg 1 740
cttttgcaact tctttaatga ca tttgaaat cgtaaatgtc ttaccggttc ctgtcgcccc 1 800
aagcaacact tgctttttct tt ccactatt aattccctct acaagcttct ctatagctac 1 860
cggctgatca ccttgcgggg aat acgctga gacaatttca aat tgacg 1 908

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<210> 20  
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 <213> Murine

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 1 5

<210> 21  
 <211> 9  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> PCR Primer

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 Ser Pro Ser Tyr Ala Tyr His Gln Phe  
 1 5

<210> 22  
 <211> 25  
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 <213> Artificial Sequence

<220>  
 <223> PCR Primer

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<210> 23  
 <211> 42  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> PCR Primer

<400> 23  
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<210> 24  
 <211> 43  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> PCR Primer

<400> 24

caaggagagg atagttaact gaaaaagacc taaaaaagaa ggc 43

<210> 25

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 25

tccccgtgtc ctataattgt tagctc 26

<210> 26

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 26

gtggacggca aagaaacaac caaag 25

<210> 27

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 27

gttcctataa ttgttagctc atttttttc 29

<210> 28

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 28

ctctgtgacc tcctttgatt agtatattc 29

<210> 29

<211> 36

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR Primer

<400> 29

caatggatcc ctcgagatca taatttactt catccc 36

<210> 30

<211> 32

<212> DNA

<213> Artificial Sequence

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ggtgctcgag tgcggccgca agctt 25

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cgattccct agttatgttt accaccaatt tgctgca 37

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gcaaatggt ggtaaacata actaggggaa t 31

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agtccaagtt atgcatatca tcaattt 27

<210> 35  
<211> 33  
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<220>  
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cgatagcca agttatgcat atcatcaatt tgc 33

<210> 36  
<211> 34  
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<220>  
<223> PCR Primer

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gtcgcaaatt gatgatatgc ataacttgga ctat

34

<210> 37  
<211> 9  
<212> PRT  
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Thr Pro His Pro Ala Arg Ile Gly Leu  
1 5

<210> 38  
<211> 25  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> PCR Primer

<400> 38  
ctgtgctttg cgaatggaaa gaagc

25

<210> 39  
<211> 43  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> PCR Primer

<400> 39  
gttttcattc atacacttag acaagcgttg gcttttgac ttc

43

<210> 40  
<211> 24  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> PCR Primer

<400> 40  
gacaagcgtt ggcttttgca cttc

24

<210> 41  
<211> 44  
<212> DNA  
<213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR Primer

&lt;400&gt; 41

caaaagccaa cgcttgtcta agtgtatgaa tgaaaaccga gtgg

44

&lt;210&gt; 42

&lt;211&gt; 25

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR Primer

&lt;400&gt; 42

aagtgtatga atgaaaaccg agtgg

25

&lt;210&gt; 43

&lt;211&gt; 28

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR Primer

&lt;400&gt; 43

catataaagg ttccacaatt gccttttc

28

&lt;210&gt; 44

&lt;211&gt; 28

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR Primer

&lt;400&gt; 44

gaagcagaaa tgaagccaat actcaatc

28

&lt;210&gt; 45

&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; PCR Primer

&lt;400&gt; 45

ggttccacaa ttgccttttc aataatc

27

&lt;210&gt; 46

&lt;211&gt; 6

&lt;212&gt; PRT

&lt;213&gt; Bacillus anthracis

&lt;400&gt; 46

Lys Val Val Lys Asn Lys

1

5

<210> 47  
 <211> 12  
 <212> DNA  
 <213> Bacillus subtilis

<220>  
 <221> misc\_feature  
 <222> 5, 6, 7, 8  
 <223> n = A,T,C or G

<400> 47  
 gaacnnnnngt tc

12

<210> 48  
 <211> 331  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> PCR Primer

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 Met Lys Lys Ile Met Leu Val Phe Ile Thr Leu Ile Leu Val Ser Leu  
 1 5 10 15  
 Pro Ile Ala Gln Thr Glu Ala Lys Asp Ala Ser Ala Phe Asn Lys  
 20 25 30  
 Glu Asn Ser Ile Ser Ser Met Ala Pro Pro Ala Ser Pro Pro Ala Ser  
 35 40 45  
 Pro Lys Thr Pro Ile Glu Lys Lys His Ala Asp Glu Ile Asp Ser Pro  
 50 55 60  
 Ser Tyr Val Tyr His Gln Phe Ala Ala Asp Gln Ala Arg Glu Leu Ile  
 65 70 75 80  
 Asn Ser Trp Val Glu Ser Gln Thr Asn Gly Ile Ile Arg Asn Val Leu  
 85 90 95  
 Gln Pro Ser Ser Val Asp Ser Gln Thr Ala Met Val Leu Val Asn Ala  
 100 105 110  
 Ile Val Phe Lys Gly Leu Trp Glu Lys Thr Phe Lys Asp Glu Asp Thr  
 115 120 125  
 Gln Ala Met Pro Phe Arg Val Thr Glu Gln Glu Ser Lys Pro Val Gln  
 130 135 140  
 Met Met Tyr Gln Ile Gly Leu Phe Arg Val Ala Ser Met Ala Ser Glu  
 145 150 155 160  
 Lys Met Lys Ile Leu Glu Leu Pro Phe Ala Ser Gly Thr Met Ser Met  
 165 170 175  
 Leu Val Leu Leu Pro Asp Glu Val Ser Gly Leu Glu Gln Leu Glu Ser  
 180 185 190  
 Ile Ile Asn Phe Glu Lys Leu Thr Glu Trp Thr Val Leu Gln Glu Leu  
 195 200 205  
 Asn Val Thr Val Arg Thr Ser Ser Asn Val Met Glu Glu Arg Lys Ile  
 210 215 220  
 Lys Val Tyr Leu Pro Arg Met Lys Met Glu Glu Lys Tyr Asn Leu Thr  
 225 230 235 240  
 Ser Val Leu Met Ala Met Gly Ile Thr Asp Val Phe Ser Ser Ser Ala  
 245 250 255  
 Asn Leu Ser Gly Ile Ser Ser Ala Glu Ser Leu Lys Ile Ser Gln Ala  
 260 265 270  
 Val His Ala Ala His Ala Glu Ile Asn Glu Ala Gly Arg Glu Val Val  
 275 280 285  
 Gly Ser Ala Glu Ala Gly Val Asp Ala Ala Ser Val Ser Glu Glu Phe  
 290 295 300  
 Arg Ala Asp His Pro Phe Leu Phe Cys Ile Lys His Ile Ala Thr Asn



```

305          310          315          320
Ala Val Leu Phe Phe Gly Arg Cys Val Ser Pro
          325          330

```

```
<210> 49
<211> 8
<212> PRT
<213> Gallus gallus
```

<400> 49  
Ser Ile Ile Asn Phe Glu Lys Leu  
1 5

```
<210> 50
<211> 9
<212> PRT
<213> Homo sapien
```

```
<400> 50
Val Leu Gln Glu Leu Asn Val Thr Val
 1               5
```

```
<210> 51
<211> 9
<212> PRT
<213> Homo sapien
```

```
<400> 51
Tyr Leu Ser Gly Ala Asn Leu Asn Leu
  1             5
```

```
<210> 52
<211> 9
<212> PRT
<213> Artificial Sequence
```

<220>  
<223> PCR Primer

```
<400> 52
Tyr Leu Ser Gly Ala Asp Leu Asn Leu
 1             5
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